

Geneva may be a can of worms

Last week's meeting at Geneva has committed the Soviet Union and the United States to solve three huge problems in arms control which have individually been insoluble. Is that wise?

THE outcome of last week's meeting between the United States and the Soviet Union is a disappointment. The occasion may yet turn out to have sown the seeds of greater danger. For, on the face of things, the agreement between Mr George Shultz and Mr Andrei Gromyko that there should be three interlocking parallel negotiations on strategic arms control (long-range missiles, intermediate missiles and anti-missile systems) is a recipe for a repetition of the breakdown at the end of 1983 of the earlier negotiations on strategic arms. The two men have in effect promised to build an exceedingly ambitious house of cards. The obvious danger is that the whole edifice will come tumbling down long before it can be completed, as one side takes the view that the other has done something to jeopardize the spirit of the negotiations due to begin in March or thereabouts. Shultz and Gromyko said last week that they will meet again if that should be necessary; it will be safest if they can do so before March, to anticipate the stumbling blocks that lie ahead.

The general but contrary opinion, that last week's meeting was a triumph for rationality as well as for those who took part in it, stems from the belief that it is something to be grateful for that the Soviet Union and the United States are willing to talk to each other. There is something in that view. It is also no doubt a plus that neither side saw fit to storm out of last week's two-day meeting. But a decision to open negotiations is to be counted as an achievement only if there is a reasonable chance that negotiations will not be a prelude to another falling out.

Past lessons

The events of 1983 are all too vivid a pointer to the trouble that may lie ahead. Then, there were two sets of parallel negotiations under way, on strategic missiles and missiles of intermediate range. The Soviet Union had been complaining for several months at the plan to install US cruise missiles and Pershing II rockets in Western Europe, a formal decision taken by the North Atlantic Treaty Organization four years earlier in response to Soviet deployment of SS20 missiles during the late 1970s. With the arrival of the first cruise missiles in Britain, the Soviet Union pulled out of the talks on intermediate-range missiles, but the parallel negotiations continued for a couple of weeks, suggesting to the optimists that they might resume in 1984. But that was not to be. At least to the Soviet Union, the linkage (the fashionable descriptor) between the two projects was too strong. One consequence was a deterioration of superpower relations that kept many people awake at nights throughout George Orwell's year. Another was that originally tentative plans for military developments became entrenched. The most notable of these, the United States plan to develop a defence against strategic missiles, the strategic defence initiative (see page 177) as it is called, emerges from last week's talks as the most likely candidate occasion for an angry repudiation of the negotiations now to begin.

What seems to have eluded Shultz and Gromyko last week is agreement on the procedures that superpower negotiations on arms control must satisfy to succeed. Such a mechanism must be sufficiently robust to survive the recurrent bouts of superpower bad temper that have been a feature of the past four decades, and which must prudently be assumed to have become perpetual. The objective in arms control should be a set of agreements on strategic issues capable of surviving all disagreements except those

likely to persuade one side or the other that a nuclear war is a lesser risk. The belief is mistaken that arms control agreements by themselves may engender such a state of sweetness and light that serious conflicts of interest will no longer arise. Some benefits of that kind would no doubt accrue, just as modest agreements would pave the way for more ambitious deals. But the serious objectives are that by voluntary agreement the two sides should so limit their own strategic power that both their capacity and their inclination to embark on nuclear warfare will be diminished. That, after all, is the only reason why others than the superpowers have a legitimate interest that some kind of agreement should be reached.

Criteria

The difficulty in the present circumstances is that there seems to be no agreement between the superpowers on the general principles on which their three parallel negotiations should be conducted, or on the criteria that should be satisfied by particular agreements. To what extent, for example, should the details of an agreement on strategic arms be capable of verification, either by remote methods such as reconnaissance satellites or by the provision of more immediate access? The United States has traditionally been the more zealous advocate of comprehensive verification, the Soviet Union has recently surprised some of its critics by a willingness to entertain access (in connection with the proposed treaty of chemical weapons), but by no means can verification be watertight (the US Congress notwithstanding). To what extent should agreements not to deploy weapons of some specific kind require of its parties an interdiction of research? Deployment can in principle be verified, but research as such is necessarily uncontrollable, even in many circumstances by the governments concerned (and so cannot be realistically constrained); but what can be said about the intermediate ground of testing (which happens to be relevant to the US strategic defence initiative)? And what, in any case, is the condition of strategic stability at which the superpowers are now aiming? The capacity for mutually assured destruction, the distant (and possibly mythical) prospect of assured invulnerability, or something in between? To embark on detailed negotiations when the objectives are not clear is far from wise.

The most obvious deficiency of last week's agreement is therefore the lack of some agreed procedure for arriving at a common understanding on these questions. The issues are not matters that can be negotiated, but are partly technical, partly military, largely political but in an important sense are also philosophical. How much certainty is needed for verification to be counted adequate, for example? To what extent would the mutual disclosure of information on novel weapons enhance security? And how could that be verified? Part of the enthusiasm for last week's meeting at Geneva derived from the notion that the Soviet Union and the United States were contemplating an "umbrella" (to use their word) for a serious and continuing process of negotiations on arms control, necessarily lasting for several years. What has emerged is a formula for detailed negotiations on three outstanding issues with the implicit danger that, if one fails, none will succeed. Mr Shultz and Mr Gromyko now must provide that themselves, by means of regular meetings to assess which way the wind is blowing. □



Japanese originality

Can Japan shake off the slur of imitativity without reforming the universities?

THE Japanese are once again worrying about their supposed lack of creativity (see p.173), this time not simply because uncharitable Westerners have been pointing out that the Japanese did not actually invent the video-cassette recorder. Rather, an analysis of publications in international science journals (albeit a tiny sample) suggests that, in basic research, an enormous gulf still separates Japan from the United States. Although the white paper from the Science and Technology Agency charts quite a few differences between Japan and other countries that may help to account for Japan's poor performance, the uncomfortable feeling persists that Japan is not really doing as well as it should. Japan, after all, is spending on basic research 35 per cent of what is spent in the United States, but its people's output of scientific papers is an order of magnitude less.

Many factors may be to blame, of which one must be the lack of a tradition of scientific excellence. Language is also a severe handicap; even a Japanese who has lived abroad will be lucky if he can read scientific English a quarter as fast as a native speaker. Even so, in Japan as elsewhere, success is likely to be a function of the product of talent and resources; if clever people have the right facilities, they will do good work. But in Japan, resources may not effectively reach the talent that there is. The Ministry of Education, Culture and Science (MESC, the principal support agency for basic research) distributes its research grants with the help of some 800 advisers in the numerous committees of its Science Council. The advisers are, by and large, the grand old men of Japanese science, selected as the most esteemed representatives of the various academic societies that advise the ministry. Although many have done great things in their day, not all are on top of the latest developments. Yet a well-cultivated connection with a figure on the relevant grants committee may serve an applicant as well (or perhaps better) as the quality of the grant proposal itself. The system of forming close relationships between junior and senior figures, and the patronage it produces is, however, thoroughly Japanese: suggestions that it should simply be replaced by a more open system of assessment is received with much the same horror as the suggestion that hamburgers should replace raw fish as the national dish.

The real losers are those talented people at lesser known universities who have not been able to cultivate powerful sponsors in Tokyo. The unknown are not altogether forgotten, however. There is a countervailing tradition of Japanese democracy that nobody should be entirely ignored. The result is that a huge number of small grants (less than US\$10,000) are given out to all and sundry, which leaves very little for the really big grants that could give a group international status. Special promotion grants, worth on the average about \$350,000 a year and specially aimed at research "likely to produce outstanding results", are given to only six groups a year. But, to be fair, these grants are still quite new. They show that MESC is making some first efforts to concentrate its resources where they can be most effective. The ministry is also taking some steps to give freedom and money to exceptional young people. This year, postdoctoral fellowships — just 200 of them — will be available for two-year periods, and there are hopes that the numbers can be increased.

These developments do not, however, satisfy the other ministries and industry, frustrated by the slow pace of change at the universities. The Science and Technology Agency is pumping growing sums of money into its own research institutes, some of which carry out basic research in fields such as molecular biology, lasers and heavy-ion physics which is no different from that at universities. And industry, disappointed at the unwillingness of the universities to take on contract work (where MESC is also beginning to make reforms), is setting up its own academic research institutes, raising the prospect that there may eventually be several parallel organizations for basic research — with no major changes at the universities. The Science and Technology

Agency is too polite to suggest reforms to the Ministry of Education, but the trends noted in the report, and the slowness of change in the Ministry of Education, should be of serious concern to the universities. □

Attorneys' comeuppance

A judge has questioned lawyers' fees, perhaps making it unprofitable for them to ape vultures.

THE ambulance-chasing lawyer is not unique to the United States, but the American legal profession consistently shows a special genius in finding profit in the misfortune of others. Last month's tragedy in Bhopal brought this home with the sight of squadrons of US lawyers descending upon the worst-ever industrial accident. That the Bhopal victims have a cause of action is undeniable. That they need American lawyers, armed with contingency-free contracts, to secure justice is less certain. And it is less certain still that ambulance-chasing lawyers could even exist if the US legal system were in proper shape.

The United States has seen an explosion of personal injury claims in recent years, some worthy and some not. The social cost is enormous — vast sums of money are spent on legal research, the preparation of tons of documents and sparring over technicalities. A rational society would institute compensation schemes, but when a number of US states attempted to institute no-fault automobile insurance, the greatest opposition came from those who have profited most from the *status quo* — the lawyers.

Sceptics need only to look in the classified telephone directory of any American city to taste the flavour of legal entrepreneurship. Full-page advertisements are common. "ACCIDENT CLAIMS? SEE A LAWYER" reads one. Another provides a sort of shopping list of possible grounds for a lawsuit: medical malpractice, airplane crashes, defective products, dangerous premises and "other accidents and serious injury cases". One entrepreneur offers free initial consultation either "in your home or in your hospital room".

The American tradition in personal injury cases is that attorneys' fees are negotiated between the client and his attorney; it is rarely possible to collect directly from the losing defendant. The result is that the courts have nothing to say about fees. The exceptions are class actions, the aim of which is the creation of a fund to compensate not just an attorney's clients but anybody else who has suffered the same injury. Under federal court rules, it is up to the court to determine fees in such cases, charging them out of the fund or, in some instances, against the defendant. Last week, a federal court in New York exercised this right with a vengeance — this time against the plaintiffs' attorneys. It sent a clear message that the day of the windfall may be over.

The case was the Agent Orange class action, the suit brought by thousands of Vietnam veterans who claimed serious and persisting health damage from their wartime exposure to defoliants. The chemical companies named as defendants, while not admitting liability, had agreed to settle for \$180 million. In other class actions, courts have been quite generous to the winning attorneys. Not only have they received hourly fees and expenses, they have also often been awarded "multipliers" of hourly fees to compensate them for the risks of taking on the case. On one occasion, a court awarded four times the usual attorneys' fees. Last week, however, the court did something different. It thoroughly dissected the fee claims of the 100 attorneys involved, rewarding those it felt had made an exceptional effort on behalf of claimants (but in no case awarding fees of more than \$150 per hour and a multiplier of 1.5), and throwing out vast chunks of the expenses submitted by others.

The lawyers involved are crying foul. One said "it's going to drive competent counsel out of this area". Not everybody will consider that a loss. On the contrary, it may be a modest first step towards driving all counsel out of personal injury litigation, which process will be complete only when statutes providing for administrative compensation of injuries replaces the current relics of common law. □

European synchrotron

Confusion about site deepens further

THE need for a European scientific institution with supranational powers of decision has become even more apparent with the confusion over the site for the proposed European Synchrotron Radiation Source (ESRS). This 5-GeV intense light source is almost certain to be constructed in Grenoble near the French Alps, but there is still no sign of a formal international agreement on the matter.

Even French government sources describe the situation as "confused", although there is still confidence in Paris that eventually all will go in favour of Grenoble. Meanwhile, however, the slighted French city of Strasbourg in Alsace has gone to law against the Grenoble "decision"; and the international commission set up by the European Science Foundation and charged with finding a site for ESRS is continuing visits to possible sites, most recently Trieste (proposed by Italy) and earlier Risø (Denmark).

Paris is watching these visits with a certain degree of surprise, believing the matter to have been effectively closed by the joint decision of France and West Germany to share most of the costs of ESRS at Grenoble and a cryogenic, high-Reynolds-number wind tunnel near Cologne and, moreover, believing that the international commission is essentially powerless, as it was given no exact terms of reference.

Three main issues are outstanding. Strasbourg's case against the French government is based on the text of contracts signed between the government and Alsace in November 1983, in which the government promised to develop Strasbourg as a "European centre" (as part of the government's overall regional strategy). The text says at first ambiguously that "the French government will support Strasbourg in its negotiations with its European partners", but continues with a list of five examples to which, by implication, the "support" might be supposed to apply. Among them is the European Synchrotron Radiation Source. At that time, however, the Grenoble site had not been proposed, and it proved finally to be more appropriate, according to the French research minister. Fiercely determined to press its case, the Alsace regional council has put the facts before the Conseil d'Etat, France's highest court, but it is unlikely to win. Moreover, Alsace is in opposition hands and an Alsatian victory would be severely painful to an already battered administration.

After Strasbourg, there is the international commission, which might establish a consortium of small states to join the Grenoble ESRS as a body.

Finally, there is the question of cash and management of ESRS, which Paris sources describe as the real "international question". The Grenoble "site" now appears to be in three parts, and a steering committee is required to work out detailed site assessments and finalize a design. The French government believes that the steering committee could be established with only French and German members. West Germany disagrees, but seems likely soon to come under French pressure. West

Germany is keen to begin work on the wind tunnel — but France may insist that the pace of work on the tunnel be at least matched by work on ESRS. And since the West German government proposed the wind tunnel/ESRS package in the first place, it may be forced to agree and so set up a purely binational steering group for ESRS in Grenoble.

Thus it is possible that detailed design of a Grenoble ESRS could begin in just a few weeks. West Germany and France have between them offered two-thirds of the capital cost of ESRS, and while the other third must come from other partners such as the offended Italy and Denmark, or impecunious Britain, the steering committee would be expected to have up to two years of work ahead of it before that money will be required. **Robert Walgate**

US space

Commercialization set back

Washington

THE National Aeronautics and Space Administration (NASA), while announcing new incentives to attract commercial investment in space, is acknowledging that any profits to be had in space manufacturing will be a "long-term proposition". The NASA official appointed to head the agency's new office to promote commercialization, Isaac Gillam, has injected a heretofore absent note of caution in NASA's grand plans for space commerce. At a meeting organized last week in Washington by a Cambridge, Massachusetts, consulting firm that hopes to sell analysis, advice and financial services to would-be space investors, Gillam said it is "extremely important that we do not oversell the effort" at this stage, adding that he doubted there would be many successful new space business in this decade.

To overcome the most obvious barrier to space investment — the high cost of launches — NASA has taken some drastic steps, offering to shoulder many of the start-up costs for private companies willing to get into the space business. Under the new NASA policy, it will offer free rides to private companies during the research phase of their work, and may even put up several million dollars as "seed money" for selected research projects. NASA is also formulating plans for offering purchase agreements for certain space products.

NASA has already entered into "joint endeavors" with McDonnell Douglas and Johnson & Johnson, which hope to manufacture an undisclosed hormone using electrophoresis in space; John Deere, the farm machinery company which hopes to carry out experiments on new alloys; and 3M Company, which on a series of flights over the next year will study production of organic crystals.

Initial results of experiments in space have been mixed. Although 3M's one experiment was reported to be an "un-

qualified" (and unelaborated) success, the McDonnell Douglas work has been plagued with difficulties. On the recent flight of the shuttle *Discovery*, which carried McDonnell Douglas's own astronaut to run the electrophoresis apparatus, bacterial contamination in the equipment apparently got out of hand; bacterial endotoxins deactivated the product. The experiment is now being rescheduled for next summer. The company had hoped to fly a production-scale unit, with 24 times the capacity of the experimental one, next summer; that will have to be postponed until the end of next year at the earliest.

According to McDonnell Douglas scientists, the apparatus was sterilized two days before being loaded on the shuttle for launch, and the damaging bacterial growth apparently occurred during the week-long mission.

The 3M Corporation is the most enthusiastic so far. It recently submitted an elaborate plan for a series of experiments that would fly on 72 shuttle missions over the next ten years. Under the proposal, NASA would foot the bill during the research phase; in return, 3M would agree to make public its research findings within specified periods. Data from experiments on growing thin crystalline films would be released in two stages, 50 per cent immediately and the balance within a year; data from later experiments would be published within three years of the flight.

NASA meanwhile is preparing to show that at least something can be made quickly in space that someone might want to buy: in a few months it will begin to market the 20 micrometre latex spheres that it has been cranking out on recent shuttle flights. NASA administrator James Beggs said last week that the potential market for the spheres is \$100 million per year, a suggestion received with a certain scepticism as the only certain application is in calibration of microscopes. **Stephen Budiansky**

Weapons in space

Soviet alarm about star wars

Washington

SOVIET alarm about the US strategic defence initiative (SDI), or "Star Wars", is the apparent explanation for an unusual document on directed energy weapons now being made widely available through Soviet sources. The document* is credited to the Committee of Soviet Scientists for Peace against the Nuclear Threat and is available in a good English translation. After a sombre review of the potential of a variety of directed energy weapons, the report concludes that the deployment of space-based anti-missile systems would increase the incentive for pre-emptive first strikes and worsen the danger of wrong decisions in a political crisis. It would also, according to the report, stimulate the proliferation of alternative delivery systems such as cruise missiles, which may be less vulnerable to space-based attack.

The two coordinators of the report are Academician R.Z. Sagdeyev, director of the Institute of Space Research, and Dr A.A. Kokoshin, deputy director of the Institute of USA and Canada Studies, both at the USSR Academy of Sciences. A dozen or so "experts" are also credited; some are better known, however, for their political than for their scientific achievements. Copyright is claimed by the Institute of Space Research.

The report concludes that the hydrogen fluoride (HF) chemical laser has the greatest potential for use against ballistic missiles in the next few years. Referring to scientific reports in the open literature, it concludes that a basic space-based anti-missile system (SBAM) with 5-MW lasers

*A Space-based Anti-missile System with Directed Energy Weapons: Strategic, Legal and Political Implications.

using 4-metre focusing mirrors is within present technological capability and could be deployed within 8 to 10 years. Free-electron and excimer lasers are not yet sufficiently developed but might be useful by the year 2000, if powered by space-based nuclear reactors. The nuclear-pumped X-ray laser, however, is thought to have little potential; calculations suggest it might be able to focus sufficient power to destroy a missile at about 3 km — "not much above the effective range of the nuclear explosion itself". The aiming system for such a device would have to be tested in space in defiance of the existing anti-ballistic missile treaty.

Charged particle beams are quickly dismissed because they are bent by the Earth's magnetic field; of more use might be a beam of neutral hydrogen atoms produced by charge-exchange from an accelerated beam of H^+ ions. But the energy of ions required, around 1 GeV, would need accelerators several hundred metres long. Although the pointing accuracy required for a directed energy weapon has already been demonstrated in non-military applications, the need for high speed — 0.1 seconds on each missile — would be an entirely new technical challenge.

Despite the feasibility of deploying HF lasers within 10 years, the report's discussion of the logistics of an anti-missile system suggests that the technology for anything approaching complete effectiveness is still many years away. A system with 18 stations in polar orbit using 5-MW lasers, needing 126 shuttle missions for deployment, would be able to hit only 15 missiles in 100 seconds. The more powerful

800-tonne lasers that would increase this figure to 1,000 missiles are beyond present technology, according to the report. Particle accelerators, which would weigh more than 1,000 tonnes, appear to be even farther away. The cost of a feasible though far from perfect system employing 800-tonne lasers is put at up to \$2 million million, in agreement with US estimates.

The report lists a number of counter-measures that might increase the cost beyond this: they include ablative coatings on missiles, false missiles, obstacle clouds placed in the orbit of an anti-missile station; space mines kept in orbit close to the station; "sprint" missiles that travel through the atmosphere at up to 5 km per second; and ground-based lasers, which do not suffer from the same size and weight restrictions. The vulnerability of anti-missile systems is said to add to their destabilizing effect, and the development of countermeasures is (obscurely) said to increase likely civilian casualties in the event of a nuclear exchange.

As if all this were not enough, the report ends with a tug at the reader's social conscience: the funds used to develop an anti-missile system could be better used for "peaceful purposes" to benefit mankind. And — scientists please note — further development of anti-missile technology would make scientific collaboration in space impossible.

Nature's copy of the report was obtained from the Soviet Embassy in Washington.

Tim Beardsley

Pharmaceuticals

New institute

Washington

FIDIA SpA, the Italian pharmaceuticals company, has announced its intention to provide \$62 million over 20 years to establish a new neurosciences institute at Georgetown University in Washington DC. The agreement is one of the largest ever corporate/university research ventures. The institute would be staffed by Georgetown University faculty and would house fundamental research in neuro-anatomy and physiology as well as neuropharmacology.

Fidia says it already devotes 20 per cent of its income to neuroscience research in Italy, and that the new institute would gain it a foothold in US science and provide academic exchanges with Fidia researchers. A spokesman stressed that there would be no restrictions on the free and timely publication of research results from the new institute, which is to be called the Fidia Georgetown Institute for the Neurosciences. There are plans for 25,000 square feet of laboratory space.

The post of director of the institute has been offered to Dr Erminio Costa, now at Saint Elizabeth's Hospital, also in Washington DC.

Tim Beardsley

Status of peace document in doubt

THE document issued by the Committee of Soviet Scientists for Peace against the Nuclear Threat, although ostensibly a spontaneous initiative, clearly has high official approval. The official Tass and Novosti news agencies have given it wide publicity, and Academician R.Z. Sagdeyev and Dr A.A. Kokoshin are both highly placed in the Soviet hierarchy. Kokoshin was at the centre of a diplomatic row last year when the Canadian government refused him an entry visa to attend a scientific conference. The technical detail in the report is, moreover, of a kind that would normally have been classified as militarily sensitive in the Soviet Union. Although ostensibly only an analysis of options open to the United States, the document draws on data that must have been accumulated by the Soviet military.

During the past eighteen months, the Soviet Union has produced a number of special interest "initiatives" in the defence

of peace, among which the scientists' and physicians' committees are the most significant. How far such appeals are orchestrated is a delicate question.

What is clear, however, is that even when a Soviet scientist wishes to speak out against nuclear weapons, he cannot with impunity attack his own country's policy.

Last week, Academician Vitalii Goldanskii published an article supporting what he called the "noteworthy initiative" of the US Nobel laureate Glenn Seaborg, who has advocated the signing of a complete nuclear test-ban to commemorate the 40th anniversary of Hiroshima and Nagasaki. Goldanskii found it necessary, however, to begin by asserting that "it is the United States that is above all to blame" for the lack of a comprehensive test-ban treaty, and to condemn Washington's claims that "it is impossible to ensure effective control" over such a treaty.

Vera Rich

Danube Commission

Rash of riparian rows

THE Austrian government has given in to ecological pressure and the Czechoslovak government by halting the clearing of land for the proposed Hainberg hydroelectric dam in the Au forest. The Czechoslovak government has for some time been complaining that the dam, on the Danube, would lower the water table and cause considerable economic and ecological damage downstream. But the dam is only the latest of a series of squabbles about the proper use of the Danube.

Only last week, Belgrade radio was complaining that, during the present cold spell in Central Europe, Romania has been "appropriating" some 5 million kilowatts of hydroelectric capacity from the Danube, decreasing the water level by 2 metres and forcing the Yugoslav power station at Kostolac to close down. And last November, shipping on the Danube was brought to a halt when Romania closed the locks of the Iron Gates for maintenance, giving only a few days' notice rather than the minimum of two months required by the 1984 Danube Convention.

The deficiencies of the convention, and of the International Danube Commission which administers it, are thus being exposed. Part of the trouble is that the convention is concerned only with navigation on the Danube. The original members of the convention were the riparian countries from Czechoslovakia downstream, with the Soviet Union represented twice (as the USSR and the Ukrainian SSR). Austria acceded in 1960 but there has never been a serious suggestion that West Germany should sign, since the river is not commercially navigable until it reaches Austria.

Although the Danube Commission's competence has been extended to include, for example, pollution due to shipping and the regulation of barges carrying hazardous chemicals, it has no mandate to deal with other sources of pollution.

In the dispute over the Hainberg dam, both Austria and Czechoslovakia have been able to claim that they have been acting in accordance with the commission's ruling. Austria, for example, has said that it is carrying out the commission's resolution that all member states should accelerate development of the Danube waterway by building dams which, to be cost-effective, must be hydroelectric stations. (Austria has renounced the nuclear option, and so also needs the electricity.) Czechoslovakia, on the other hand, says that Austria's "unilateral" decision to build a dam at Hainberg "violates the tried and trusted concept of the comprehensive use of the Danube" developed by the commission. Both governments claim ecological support — the Austrians that the dam would prevent upstream deepening of the Danube

channel and the consequent desiccation of important wetlands, Czechoslovakia that the downstream channel would be deepened.

Ironically, the Czechoslovak argument that the water table would be lowered is reminiscent of that advanced by Hungarian ecologists against the joint Czechoslovak/Hungarian project to build two dams on the Danube, at Gabčíkovo in Slovakia and Nagymaros in Hungary. The second of these, which would drown the scenic "Danube bend" above Budapest, has been suspended since 1981, ostensibly for lack of funds. But the Czechoslovak side has forged ahead at Gabčíkovo, ignoring the claim of the Hungarian ecologists that the water table in northern Hungary would be lowered. Especially

because Gabčíkovo would divert the main navigation channel away from the Hungarian frontier into Slovak territory, it is clearly a matter within the competence of the Danube Commission.

The commission has nevertheless been strangely silent, perhaps because, from its seat in Budapest, it is reluctant to embarrass its Hungarian hosts. In the past few days, however, the commission has been able to set up a joint Czechoslovak/Austrian team of experts to assess the ecological consequences of the Hainberg scheme, while the Austrian Chancellor, Mr Fred Sinowatz, is said to have promised to pay more attention to ecological matters in future.

The Danube Commission has thus been spared embarrassment for the time being, but if it is to retain its credibility for competence and effectiveness, some rethinking of its role seems an urgent need.

Vera Rich

US tax deductions

There's no place like home

Washington

A US APPEALS court, overruling the Internal Revenue Service (IRS), has allowed a university professor to deduct from his income tax the cost of maintaining an office in his home. The ruling is expected to relax significantly what had been a virtual prohibition by IRS against such deductions by faculty members.

Tax rules allow employees to claim a deduction for an office in the home if three conditions are met: the office must be used exclusively for business and on a regular basis, it must be the employee's "principal place of business" and it must be maintained for the "convenience of the employers". It was the last two points that were in contention in the appeals-court case, which was brought by David Weissman, a philosophy professor at the City College of New York.

In ruling in Weissman's favour, the court explicitly found that a professor's duties include research. Weissman said he spent 80 per cent of his working hours at home, doing scholarly research. IRS argued before the court that most of Weissman's research had nothing to do with his job, but was merely to "increase his own prestige" and to secure "a more lucrative position". IRS had prevailed with that argument in the lower US Tax Court, which sided with IRS in a decision last year.

The appeals court, however, noted that City College by-laws require promotions of faculty members to be based in part upon a professor's "record of significant scholarly achievement". And it agreed with Weissman that his principal place of business was in fact his home. The court cited its own earlier finding that a group of musicians who performed at Lincoln Center in New York City were entitled to deduct the expense of maintaining practice

space in their homes: "A college professor's principal place of business is not necessarily the college at which he teaches any more than a musician's principal place of business is necessarily the concert hall in which he performs".

Many faculty members were able to claim home-office expenses on their tax returns before 1976, when Congress tightened the requirements; under the old rules, if such an office was "appropriate and helpful" to an employer, it qualified. The new ruling does not turn back the clock that far and may not be applicable to other professors who want to claim similar deductions.

For one thing, under the peculiar rules that apply to the tax-court system, the court's decision sets a precedent only in the circuit covered by this one appeals court, which includes New York, Connecticut and Vermont. The lower Tax Court's decision, though overturned in this one circuit, is still the law in the rest of the country. IRS would thus be within its rights to continue disallowing similar claims from professors in most of the country. In addition, a successful claim may have to show that the university did not provide adequate office space, making a home office a virtual necessity. The appeals court seemed to be impressed by the fact that Weissman's office at City College was shared with several other faculty members, had no typewriter and was the target of frequent break-ins and vandalism, making it unsafe for storing research material or office equipment.

The City College Professional Staff Congress, the faculty union, hailed the ruling as a "victory" that "clearly states that a faculty member's responsibility involves more than teaching and classroom responsibilities". **Stephen Budiansky**

Australian space

Plan to win more business

Canberra

THE Australian Commonwealth Scientific and Industrial Research Organization (CSIRO) has set up a body to coordinate its own space and communications research. The aim is to encourage Australian industrial companies to the point where they will gain sufficient experience to compete internationally for parts of the growing satellite-based communications market. The new unit will be known as the CSIRO Office for Space Science and Applications (COSSA).

It is perhaps surprising that a country as far-flung as Australia has no central body like the US National Aeronautics and Space Administration (NASA) or the European Space Agency (ESA) to coordinate developments in communications technology. An early competence was built during the 1950s and 1960s in support of the joint United Kingdom/Australia testing programme at the Woomera rocket range and the NASA satellite-tracking network, but although Australia was one of the eleven founding members of Intelsat, during the twenty years of the consortium's operation, no Intelsat contracts have been placed with Australian companies for the supply of flight equipment. In fact, within the past year, the contract for a ground-control station was lost to Indonesia.

Although Australia will now supply the image-detection system for a new tripartite space telescope project with NASA and ESA, it had been previously thought that Australia's bids for international contracts would be spearheaded by participation in the now-defunct Starlab project, which faltered when Canada withdrew in March 1984. In the same month, CSIRO established a Space Science and Technology Study Group, which, in November, urged CSIRO to use its expertise to stimulate the industrial sector with contracts in such fields as ground sector space communications systems and remote sensing, and to collaborate in international projects such as the reusable US Spartan satellite and ESA's radar satellite ERS-1.

The local industry's share of the \$A330 million expenditure in the government's satellite-owning company Aussat amounts to about \$A63 million, with a possible extra \$A70 million through offsets. Aussat is due to have its first two Hughes domestic satellites launched from the space shuttle in July and October this year, with a third perhaps in mid-1986, to be launched by Ariane-space. By the end of 1985, Australia will have spent about \$A500 million on operational space systems such as Intelsat, Aussat and Inmarsat, but relatively little of the equipment will have been manufactured in Australia. More to the point, by 1995, the country's annual expenditure on space-related technology is estimated at between \$A370 and \$A500 million.

The directorship of COSSA will be filled by Dr Ken McCracken, at present chief of the CSIRO Division of Mineral Physics. Using Skylark rockets flown from Woomera, he was a pioneer of Australian X-ray astronomy and introduced the use of Landsat satellite pictures into the management of Australian resources.

Introducing COSSA this week, Dr Paul

Wild, chairman of CSIRO, observed that if approved by cabinet for this year's budget, COSSA would spend \$A7 million in 1985-86, rising to \$A20 million per annum at the end of the decade. In the international space market, a company could only get a contract to build part of a commercial satellite if it had done so before: no previous experience meant no contract. By letting out up to 70 per cent of the contracts for CSIRO's space experiments to Australian companies, he said, CSIRO will break this vicious circle. **Jeffrey Sellar**

Mongolia

Progress with problems

MONGOLIA's new Party leader, Dr Jambyn Batmonh, has sharply criticized the country's alleged progress in industrialization and the modernization of agriculture. Addressing the Central Committee of the Mongolian People's Revolutionary Party (MPRP) last month, he drew attention to the frequent waste of financial investment and human effort by the neglect of underlying scientific principle. His tone contrasted strongly with that of the official eulogies, only three weeks before, during the celebration of the diamond jubilee of the Mongolian People's Republic, which presented Mongolia as a shining example

completed on schedule, and there is an increasing backlog of uncommissioned facilities, due to the inadequacy of the transport infrastructure. Insufficient allowance has been made by planners for the fact that, although construction materials are (theoretically) produced at a uniform rate throughout the year, most construction and installation work can be done only between late spring and early autumn and that, just when the building season starts, there are vast amounts of other heavy goods to be moved.

In agriculture, the establishment of modern farms has not been backed up by "good and stable crops" for Mongolian conditions, nor the proper organization of pedigree stock-breeding on a "scientific basis" (a topic doubtless close to Dr Batmonh's heart, being himself the son of itinerant herdsmen). Water management is uncoordinated and, in some cases, neglect of the "features of soil fertility and the biology of plant cultivation and crop rotation" means that the construction of expensive irrigation systems has actually led to a falling-off of crop yields.

In the country's new industries, poor work at the design stage has led to high production costs, wastage of raw materials and insufficient use of equipment and machinery. Training of workers in modern techniques and production methods should be treated, Batmonh urged, as a matter of "very serious concern".

Mongolian specialists still, to a large extent, depend for advanced training on the Soviet Union (Mongolians hold first place there among foreign students), and the Soviet Union and the other Comecon countries have a number of technical co-operation projects with Mongolia, including a major geological survey of the country.

According to Batmonh, Party plans to make Mongolian industry and agriculture more productive include wider use of the "brigade organization" of labour (payment on the basis of output of a team) and "socialist competition" between workers. These are, Batmonh explained, "tried methods" for involving workers in the implementation of large-scale plans.

Vera Rich



for Third World countries wishing to make the leap direct from feudalism to a centrally planned industrial society.

Mongolia's achievements, according to the jubilee data, are impressive. Since 1966, when the fifteenth congress of MPRP adopted a plan for "completing the building of socialism", major industrial centres have been established at Darkhan, Erdenet and Baganur, with industry now accounting for more than 30 per cent of the national income. Coal production has gone up sixfold, generation of electricity more than eightfold, mining for metal ores close to 40-fold. More than 660,000 hectares of virgin land have been brought under cultivation and 20 "highly mechanized" state farms established.

Unfortunately, according to Dr Batmonh, investments have not always been properly planned to suit Mongolian conditions. Construction projects are not

Japanese research

More creativity wanted

ONCE again the Japanese government is calling for "more basic research and more creativity" in the nation's scientific research programmes.

This time it is the latest White Paper from the Science and Technology Agency that stresses the need for greater efforts. But unlike previous reports, the new paper goes some way towards explaining just why there is so much concern about creativity in Japan.

The 419-page paper, entitled *Aiming at the creation of new technology for the 21st century* takes as its starting point an attempt to assess just how well Japan's basic research is doing. This is no simple matter, and the report opts for a simple analysis of the number of papers published in important international science journals in three areas: life sciences, materials and surface sciences, and computer architecture and pattern recognition. The first groups are analysed by the number of papers published in *Nature* and up to seven other international journals, for computers two journals are used.

The results make pretty depressing reading for the Japanese. In materials and surface science, the United States is publishing 25 times more papers a year than Japan, in the computer field 6–8 times more, and in life science 5 times more. Comparison with "Europe" (here taken as the United Kingdom, West Germany and France) paints a more favourable picture: Europe produces five times more papers in materials and surface science but only about 20 per cent more in computer and life sciences. For the Japanese though, it is the comparison with the United States that really counts; Europe has long been written off as a supplier of rock music, fashion and sausages rather than a serious technological competitor.

Part — but not all — of the discrepancy between Japan and the United States is accounted for in terms of the difference in funding. Japan's total expenditure and basic research is obviously less than that of the United States but, as the White Paper shows, at 35 per cent of the US total is not so much less as might be expected. The percentage of funds going to basic research in Japan is, however, on the decline — down to 14.6 per cent in 1982 from 24 per cent a decade ago — despite endless government calls for more attention to basic research. But the trend is not so hard to explain. The bulk of the Japanese research effort (and funds) is provided by industry, where expenditure has been increasing with great rapidity. Expenditure in the universities and institutes where basic research is carried out has simply not kept pace with industrial expansion. Not surprisingly, the expanding industrial laboratories are largely concerned with applied research.

Indeed, the report also gives figures suggesting that the whole thrust of science education is away from the basic towards the applied. Japanese universities produce a staggering number of engineers — more in total than the United States, even though the actual number of university students in the United States is five times higher. In contrast, the United States and the United Kingdom produce huge numbers of graduates in the natural sciences (among graduates, the ratio of engineers to scientists is 6.4 in Japan, 0.8 in the United States and 0.6 in the United Kingdom). Not surprisingly, given the bias towards engineering, most graduates quickly make their way into industrial companies; only three per cent of Japan's students go on to further university training, whereas in the United States and Europe it is closer to 18 per cent. Indeed, although the United Kingdom has only one seventh the number of undergraduate students as Japan, the nation produces getting on for double the number of doctoral and masters' theses in science.

The report also raises worries over just how well Japan is doing in the technology

trade, thought to be a reflection of the development of "basic" new industrial processes. Overall, there is no doubt that Japan is massively in the red when it comes to payments for licences and patents. But this is not such a serious problem. Many payments are still being made for technology licensed years ago. When only recent licences are taken into account, then the beginnings of a shift towards a technology trade surplus can be seen.

How then is Japan's basic research performance to be improved? The general suggestions made in the report are familiar ones: funds to cultivate young and talented researchers, increased exchange between universities, industry and government research institutes, greater investment in the equipment and facilities necessary to back up advanced research and increased investment in personnel at the universities and national research in institutes.

If present government policy measures are anything to go by, however, the result will be an increasing basic research and role for private industry. Increased tax incentives for industry to perform basic research and joint research with industry at government research institutes are measures likely to be enacted before long, rather than increased funds for the universities.

Alun Anderson

Superconducting supercollider

States vie for US accelerator site

Washington

THE competition has begun among state governments to play host to the proposed Superconducting Supercollider (SSC). If built — which is still not formally decided — the accelerator will bring millions of dollars and thousands of jobs to the state lucky enough to be selected. And while the Department of Energy insists that the site will be selected on the best scientific criteria, many states are already letting it be known that a decision in their favour would be rewarded with financial benefits.

SSC, a proton/proton collider, would be easily the world's largest accelerator, with a circumference of between 60 and 100 miles, depending on the type of magnets used. The cost of construction, which would take 6 years, is estimated at \$3,000 million at 1984 prices. When completed, there might be 2,000 people directly employed.

Texas is making one of the strongest pitches for SSC. Governor Mark White has made it clear that the state will do whatever is necessary to present its case, including making available state funds to assist with the cost of the project. A site selection committee is now at work and has settled on four or five locations. The criteria used include proximity to a large airport, and Houston is seen as a big draw.

Research into a possible magnet design for SSC is being undertaken at the Texas Accelerator Center, which reports encouraging results with superferic magnets.

Peter McIntyre of Texas A&M University says that completed magnets of the Texas design would cost less than half those of competing high-field designs — \$350 million as against \$800–\$900 million. The catch is that superferic magnets, with a field strength of 2–3 tesla, require larger tunnels, but McIntyre is confident that the magnet cost saving is decisive.

Texas is faced with strong competition from Illinois, which has already spent \$500,000 on geological surveys to find a suitable site in the state. One strong attraction is that it might be possible to utilize the proton injector at Fermilab, with a substantial cost saving. In addition, there is already the laboratory infrastructure in place to support scientific staff during construction. Illinois also has a governor-appointed task force reviewing possible sites, and local industries have been persuaded to join in. In Colorado, \$200,000 of state funds have been spent on site selection and universities and industry are combining forces to present a case to the Department of Energy. Other interested states include Utah, Arizona, Michigan and Minnesota.

A document detailing site selection criteria is now being drawn up by the department's contractor, the Universities Research Association, and will be published in April. The race to capture SSC will then be on in earnest if construction is to start as hoped in 1988.

Tim Beardsley

Australian commission

Penney's evidence on British nuclear bombs

THE hearings of the Australian Royal Commission in London may not yet have got to the bottom of what happened during the British nuclear weapons tests in Australia, but they have told the British more about the politics of their bombs. Last week it was revealed that Sir Winston Churchill had refused in the 1950s to share British nuclear secrets with the United States, insisting on separate bomb tests at a new site in Australia.

Lord Penney (75), then Dr William Penney, in charge of Britain's test programme and the first witness for the British government, told the inquiry of a "highly restrictive" offer made in September 1951 by the United States as a condition for Britain using the Nevada test-site; all the material in the bomb and the physics involved would have had to be disclosed. Lord Penney said that he had been willing to accept the conditions to rekindle Anglo-US collaboration, but that Churchill refused.

As the first director of the Atomic Weapons Research Establishment (AWRE), Penney was under considerable pressure from the military to produce an "operational" bomb and started plans to develop high-yield weapons. Lord Penney said last week that the Australian government and Australian and British public were kept in the dark about the actual sizes of the bombs tested. The second British test explosion in the MOSAIC series, on 19 June 1956, was described by the British government as being of a size similar to the first test, that is 15 kT, but was revealed to have been a 60-kT explosion only in March 1984. A 1957 AWRE report on the tests even suggests that the yield could have been as much as 98 kT, but Penney said last week he mistrusted that figure. He explained that the most accurate measure of the bomb's yield would come from radiochemical analysis of debris from close to ground zero, revealing the amount of plutonium actually exploded, but the report detailing results from this method of yield detection has not yet been made available by the British government since it is "weapon-design sensitive".

Mr Justice McClelland was anxious to clear up a question facing the inquiry on Monday. This concerned confusion over documents released by the British government which were marked "obsolete". Mr McClelland said that he was reminded of the Watergate trial at which reports had been officially marked "inoperative". Mr Robin Auld QC spoke for the British government and assured the commission that this was simply an indication that subsequent reports exist which take into account current knowledge, rather than a revision of the actual material of the document.

Lord Penney admitted that efforts to determine the level of ground contamination after the BUFFALO tests of late 1956 were not entirely satisfactory. The commission's counsel, Mr Peter McClellan, inferred from this that similar problems were likely to have been encountered at the TOTEM tests, some three years earlier, after which "black mists" were seen in central Australia. Lord Penney said, however, that he had not even heard of the mists until he read about them in the British press two years ago. He also said that he had no knowledge of concern about the cloud from the MOSAIC 2 test.

Penney did however agree that forecast trajectories of clouds from the BUFFALO series were found to be drastically off course. One trajectory, predicted to pass north of Brisbane, was observed just north of Sydney — "not very close to Brisbane at all" as Mr McClellan put it. The cloud from the last test in the series was also predicted to travel north of Brisbane, but it was actually found to pass over most of the Australian mainland. Penney insisted, however, that the doses received by people would have been too low to cause concern.

On the safety of aborigines, Mr Geoff Eames, representing ten aboriginal organizations, challenged Penney with a 1956 AWRE report stating that "for aborigines in the tribal state, virtually naked and in bare feet . . . an acceptable level (of radiation) was only to be found at 240 miles from ground zero". Lord Penney, who had set a distance of 100 miles as being acceptable, said that he did not recall this recommendation, but that he knew of aborigines living between 200 and 300 miles from ground zero. Mr Eames told the inquiry of homesteads as close as 107, 120 and 160 miles from the Emu site and 175, 200 and 210 miles from Maralinga.

On previous evidence that aborigines were found living in the Marcoo crater after the BUFFALO series, Penney said he could not confirm or deny these reports. He said there had been an agreement between the British and the Australians that, when there were no British at the test site, site-security was Australian responsibility.

He denied that he regarded the Australians at the tests as "radiologically expendable", saying that if he had heard of aborigines living in the crater, his first question would have been "what dose have they got?". But he acknowledged that although he had advised that site areas should be marked as dangerous, by notices in various languages and "scary signs", he was certain that this had been done only after the HURRICANE tests in 1952, but not subsequently. Illiteracy seems not to have been taken into account. Penney also said that he had no recollection that soldiers were threatened with courts-

martial if any of them told the outside world that aborigines had been found in the crater, as alleged in previous evidence to the inquiry.

Throughout the British tests in Australia, the International Commission for Radiological Protection (ICRP) safety guidelines were followed, said Penney, but only after the Windscale fire in 1957 were the dangers of radiation fully appreciated. That sparked off a "revolution in health physics". Penney explained that, because some tasks involved radiation greater than the ICRP recommendations, a radiological protection branch was formed to be responsible for supervising the radiological health and safety of British weapons work.

Radiation doses were measured by film badges. The inquiry has learned that servicemen were unwilling to follow the advice to wear these close to their genitals, and that a report of experiments in 1956 showed that the result might be to underestimate exposure by 40 per cent. But Lord Penney said he could not remember revising the regulations on dose levels in the light of this report.

One of the allegations put to Penney is that he had asked for sections to be removed from a report by a member of the Australian CSIRO (Commonwealth Scientific and Industrial Research Organization) expressing concern over radiation levels from the British tests, in particular the BUFFALO series. Lord Penney was said to have seen the report as "alarmist pessimism" and scientifically unreliable as well; even after the report had been modified, publication was denied. Penney told the inquiry that he did not remember asking for sections of a report to be removed, but conceded that such a report from a man of reputation would have caused an almighty row. He agreed that if the report could have been upheld scientifically, it might have jeopardized the completion of the test programme.

Despite evidence that Australians at the test sites felt they were not consulted sufficiently, Penney said that they played a role in deciding whether or not to fire particular weapons. He said that the only time when the Australians demurred about a test was when 15 parliamentarians were going to be present to see the shot.

At Monday's sitting it was suggested that scientists working on the tests became so involved in the science that they failed to consider the implications of their work. Penney denied being carried away by the scientific beauty of the programme; "By God, I wish I could have got out and gone back to university. . . I thought we were going to have a nuclear war where the only hope I saw was that there should be a balance between East and West, but what I really wanted was to be a professor".

Susan Watts

Radiation exposure

Moving standards in 1950s

INTERNATIONAL recommendations on exposure to "ionizing radiations" were in a state of flux in the 1950s, when British nuclear weapons tests in Australia were at their height. According to Joseph Rotblat, the radiation physicist and past secretary-general of the Pugwash conferences, the central issue was the existence (or otherwise) of a threshold exposure, below which radiation would have no harmful effect.

The history was this. Britain had taken the lead (paradoxically, in the light of present Australian accusations) in radiation protection in 1921, with the establishment of the British X-ray and Radium Protection Committee — whose recommendations were first adopted internationally¹ in 1928, and promulgated by the Second International Congress of Radiology at Stockholm. At the time, those most in danger were hospital workers using X-rays and radium for treatment of patients. The known dangers were then said to be "injuries to the superficial tissues" and "derangements of the internal organs and changes in the blood". Protection measures were defined in terms of thicknesses of lead and similar materials between workers and radiation sources, rather than in amounts of radiation, although the roentgen (a measure of X-ray exposure) was introduced at the same conference.

The concepts of "tolerance" levels first appears in the international recommendations² of the Zurich radiology conference of 1934, which concluded that "the evidence at present available appears to suggest that under satisfactory working conditions a person in normal health can tolerate exposure to X-rays to an extent of about 0.2 international roentgens (r) per day... nonsimilar tolerance dose is at present available in the case of radium gamma rays". The procedural recommendations which then follow are "generally in harmony" with a 0.2 r threshold.

These recommendations remained in force, unchanged, for 16 years, through the 1939–45 war, Fermi's development of the atomic pile and the construction and use of atomic weapons. With the establishment of the International Commission on Radiological Protection (ICRP) at the sixth international congress on radiology in London in 1950, new recommendations³ were published in 1951 and were considerably more extensive than those of 1934. These were in force during the early to middle part of Britain's tests in Australia. The first exhaustive recommendations of ICRP, including limits on internal exposure to radionuclides, however, did not appear until 1954, half way through the tests.

According to ICRP's 1951 recommendations, "the effects to be

considered" were:

- Superficial injuries.
- General effects on the body, particularly the blood and blood-forming organs, for example production of anaemia and leukaemias.
- The induction of malignant tumours.
- Other deleterious effects including cataract, obesity, impaired fertility and reduction of life-span.
- Genetic effects.

Also "the previously accepted value of 1 r per week for maximum permissible exposure of external radiation itself needs revision in the light of the radiation to which workers are now exposed... the figure... seems very close to the probable threshold for adverse effects". Allowed levels were thus halved.

While thus including a reference to "thresholds", however, ICRP 1951 adds that "in view of the unsatisfactory nature of much of the evidence on which our judgements must be based, coupled with the knowledge that radiation effects are irreversible and cumulative, it is recommended that every effort be made to reduce exposure to all types of ionizing radiations to the lowest possible level".

The ICRP 1951 document also limits exposure to fast neutrons (measured in terms of energy absorbed per gram of tissue) to not more than one-tenth of that permitted for X-rays, thus introducing the concept of "relative biological effectiveness" of radiations. It was assumed that neutrons have an average tenfold greater effectiveness (in terms of damaging ion pairs per joule of energy deposited) than X-rays.

The revision⁴ of ICRP 1951 in 1954 was essentially a new document (92 pages compared with seven), much of it concerned with setting — for the first time — maximum permissible body burdens and concentrations in air and water of a large number of radioactive isotopes. ICRP 1954 also makes the first definitions of the rad and rem, radiation units still effectively in use (except for a numerical factor). The recommendations also suggest limiting the exposure "of a large population" to levels a factor of ten below those permitted for occupational exposures.

The 1954 recommendations finally laid to rest the concept of threshold. "Since no radiation level higher than the natural background can be regarded as absolutely 'safe', the problem is to choose a practical level that, in the light of present knowledge, involves a negligible risk".

ICRP 1954 also considers in much more detail than the 1950 recommendations the exposure of "critical organs". X-ray doses were limited to 0.3 r per week to the blood-forming organs, gonads and eyes (roughly a fifth the pre-war whole body value) and 0.6 r per week to the skin.

The 1954 recommendations also limit "temporary" exposure. "Since it is generally impossible to predict how long a person may be occupationally exposed to radiation", say the recommendations, "it is prudent to assume that it may continue throughout his life. Therefore "temporary" exposure at levels higher than the permissible weekly dose should not be permitted", a recommendation which might be considered to apply, for example, to soldiers and others exposed to the effects of British tests in Australia.

All these recommendations were further revised⁵ in 1958, and more recently. Current recommendations⁶ are in ICRP publication 26, published in 1978.

Robert Walgate

1. *Br. J. Radiology* 1, 359 (1928)

2. *Br. J. Radiology* 7, 695 (1934)

3. *Br. J. Radiology* 24, 46 (1951)

4. *Br. J. Radiology Supplement* no. 6 (1955)

5. *ICRP Publication no. 1*, Pergamon Press, London (1959)

6. *Ann. ICRP* 1-2 (1977–9)

Halley's comet

Japanese launch

Tokyo

JAPAN's first Halley's comet probe (MST5) was successfully launched from the Uchinoura Space Center, Kagoshima, on Tuesday, 8 January.

The satellite, given the name *Sakigake* (Pioneer), orbited the Earth until Friday and was then sent on its way towards Halley by a puff of propellant gas released on command from the control centre. It will now go on to orbit the Sun almost one and a half times before passing about six million kilometres from Halley's comet in early March 1986. Japan thus becomes the third country — after the United States and the Soviet Union — to have successfully sent a satellite into interplanetary space.

The launch came after a series of delays, caused at first by bad weather and then by a malfunctioning nozzle, but is a resounding success for the new Mu-3SII launcher.

The 138-kg MST5 satellite is itself largely a test craft for the main Planet A Halley's comet probe, which will be launched in August. But as well as testing the various new control and communication subsystems, the satellite carries a range of scientific equipment. Observations will be made of plasma waves and electromagnetic wave radiations from the comet; of the interplanetary magnetic field; and of the temperature, velocity and density of ions in the solar wind. The launch has been arranged so that when the main probe Planet A, which will be carrying a camera to take ultraviolet pictures of the comet's hydrogen coma, is close to the comet, the test satellite MST5 will be situated downstream in the solar wind from the Sun. By coordinating the two sets of observations it should be possible to understand how the solar wind "blows" the long plasma tail of the comet away from the Sun.

Alun Anderson

Laboratory animals

SIR — In dismissing Christine Stevens' proposals (*Nature* 27 September, p. 295) for remedying the wilful ill-treatment of laboratory animals in the United States, Daniel Kline tells animal welfare groups what they should do to "breach the barrier" between them and the public (*Nature* 15 November, p. 191). Mr Kline's attitude towards these groups can be shown to be patronizing and based on prejudice and naivety by recommending to all (*sic*) scientists proposals analogous to his.

First, all groups of scientists should state unequivocally that they recognize that some types of animal experiments are unnecessary and cite which they do not support. Second, they should advocate legislation which allows animals experiments to be conducted only if adequate care of the animals and supervision of facilities can be paid for. Do people who breed and use animals for sport get special government support to ensure humane treatment of the animals? And is Mr Kline implying that the current standard of care is unsatisfactory and that, unless government funds are specifically provided to improve it, scientists will carry on regardless? Third, they should actively support access of a small percentage of unbiased people — journalists, for example — to "accredited and supervised" laboratories to verify that accreditation is indeed "an ironclad guarantee of first-class care and treatment of animals".

Unless such simple steps are taken and acted upon with sincerity, the public can only conclude that scientists do approve of the development of procedures such as the burning of rabbits' eyes with shampoos. The decision that people should be given huge sums of money to torture and kill animals for the benefit of mankind was not made lightly. However, just as the decision that some shampoos should be tested on rabbits' eyes involved the searching of our consciences, there are also situations, such as the need continually to develop "better" shampoos and "better" weapons, which can be avoided. It is in the power of groups concerned with animal welfare, including, I hope, Daniel Kline and other scientists, to take the few steps suggested above to the betterment of animals as well as mankind.

Meanwhile, what should be our response to those who break current laws to liberate/steal animals from scientific research institutes? Accept for the moment that these people are misguided. At the same time, Ms Stevens has convinced Mr Kline that "some animals are being abused in medical research". In spite of the reports of veterinary inspectors of the US Department of Agriculture (USDA) showing that "Harvard, Yale, Johns Hopkins, Vanderbilt, the universities of Utah, Rochester, Pittsburgh ... repeatedly violate USDA's minimum standards" (*Nature* 27 September, p. 295), Mr Kline's

response is to say "one can only sympathize with the animals and feel indignant at those persons who are involved". How should the law-breaking researchers be punished? Often, they too try to justify their action on moral grounds; their premise is that *Homo sapiens* is "superior" to all other animals. Regardless of legislation, the public will continue to provide financial support for the wilful ill-treatment of animals and from time to time protest in various ways.

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Who's confused?

SIR — Jamie Hook, in his letter on "Confusion about Chinese names" (8 November, p.95), implies that, whereas some Chinese hyphenate their given name, Europeans only hyphenate their family name but never their given name. The fact is that Europeans rarely use a hyphenated family name. Hyphenated family names are mostly used by Europeans whose name is common in their respective countries in order to make it more easily identified. In such cases the name of the town or village where the family originated is added to the primary name, separated by a hyphen. European women sometimes keep their maiden name after marriage and place it behind their married name, separated by a hyphen.

In contrast, hyphenated given names, consisting of two words that are used together, are very common in several European countries such as France and Germany and the Scandinavian countries.

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Virus vaccines

SIR — Robert Walgate's article (15 November, p.190) contains inaccurate statements about the technical qualities of commercially available hepatitis B vaccines. In discussing a decision by the government of Taiwan to employ human plasma-derived vaccine manufactured by Institut Pasteur Production (IPP)/Sanofi (France), the article includes a claim by Sanofi that their vaccine is more effective than the plasma-derived vaccine of Merck Sharp & Dohme (United States), because the antigenic particles of the latter vaccine "lack some important polypeptides", specifically the "pre-S" region of the hepatitis B surface antigen. The report states that "the 'pre-S' region appears to be important in generating full immunity to hepatitis B".

This statement is not supported by the known scientific evidence and the published literature. The established high-level protective efficacy of vaccines that do not include the "pre-S" region show that antibodies against "pre-S" are not needed to achieve immunity against hepatitis B. Indeed, vaccines without "pre-S" antigen have been proved highly effective in inducing immunity against hepatitis B in the extensive clinical and field studies carried out during the past several years.

There is, by analogy, no scientific reason to believe that IPP's hamster ovary cell-derived hepatitis B surface antigen vaccine, now under development and containing "pre-S" antigen, would be superior to vaccines lacking the "pre-S" region. In fact, such hamster cell line-derived vaccine raises questions of safety for man since the cell is mammalian and is transformed.

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Tunes not enough

SIR — Patrick Rabbitt's review of *Genius, creativity & leadership: Historiometric inquiries* by Dean Keith Simonton (22 November, p.383) provides yet another example of computer-obsessed silliness, in particular the laborious calculations resulting in the quantification of musical creativity. The first notes of 15,618 themes by 479 composers were compared in an effort to assess their relative originality. "He examined the first-order transitional probabilities between notes in each of these note strings and related them to a (here undefined) baseline computation for all transitional probabilities between notes." The computation appears to show a trough in originality between 1750 and 1820.

The historiometricians and their computers pay little heed to the shaping influences of rhythm, harmony, dynamics or tone colour. Beethoven's Waldstein sonata opens with a C major chord repeated thirteen times, the scherzo trio of Sibelius's second symphony with nine repeated notes as does the first choral entry in Verdi's Requiem. The very different characters of these themes are determined respectively by rhythm, the tone colour of a solo oboe and by the hushed quality of *pianissimo* choral singing. One wonders how these themes would have scored for "originality" against the baseline computation. Worse still, the historiometrician appears to have missed the point that originality most often lies in what a composer does with a theme rather than in the theme itself.

Computers are clearly useful in comparing the sequences of oncogenes, growth factors and their receptors. The evaluation of originality is best left to those intuitive enough to recognize artistic and scientific genius.

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Defence initiative defended?

When a substantial part of the technical community insists that ballistic missile defence is in some sense feasible, critics of the project should look elsewhere for ammunition.

THE odd thing about the real world is that the least popular causes are advocated by the most able and the most genial people. That, if you like, is the dilemma of journalism, the profession of telling it like it is. Ask an Afrikaaner about *apartheid*, and you will discover a man who has thought more about the benefits of paternalism than you would have thought possible; some way along the line, he knows, you will agree that all those hospitals are better than no hospitals.

Last month, before Mr George Shultz set off for Geneva for his meeting with Mr Andrei Gromyko, Washington was nicely abuzz with people's introspections about star wars, otherwise known as the Strategic Defense Initiative (SDI). (Somebody, one must be sure, has written a paper advocating the use of the good word "initiative.") Naturally, nobody is prepared to say explicitly what he plans to put in the paper he will thrust into Mr Shultz's hand. But most people are willing to talk about the cause that they espouse, however unpopular it may be.

Could the United States ever hope to defend itself against an all-out attack from a force of Soviet missiles? If so, how? And how soon, given that the most cogent objection to what should be described as the scenario is that it may never work? What follows is an account of what people in Washington were saying in December. It is based primarily on conversations with four senior officials of the US administration but particularly with Lieutenant-General J. (for "James") A. Abramson, the manager of the Strategic Defense Initiative.

Naturally, one begins with the most obvious assertion: surely a perfect defence against ballistic missiles cannot be effective, even though it may be ruinously expensive. One should, of course, have known better. People in Washington are now (or were even in December) so practised at dealing with this kind of question that they had not one answer but several, much in the spirit of the defending counsel who argues that his client could not have committed the crime of which he has been accused because he has a cast-iron alibi, because he could not have wielded the instrument that caused the damage and that, in any case, he did it in self-defence.

The case with star wars is however more complicated and more substantial. It's only a research programme, right? As with all research, we cannot tell in advance what

we shall find. If we find it does not work, then so what? Nothing will have been lost. That, so to speak, is the alibi.

The intermediate arguments are necessarily more intricate. Abramson, whose office occupies an otherwise almost empty floor of a new downtown office building, not an annexe of the Pentagon, is an eloquent exponent of what might be done. Even as generals go, he has a quite formidable reputation. He came to star wars only after the project had been launched. He had just been manager of the space shuttle programme, having made his name as the man who built the F17 aircraft. One of SDI's most influential critics considers him the "best manager the Pentagon has had for years".

The general's style is not that of a tycoon, more that of an inventor. He is tall, fit, sparse and voluble, given to interspersing technical explanations with homilies on the importance of getting things right, while hunting for the right transparency in the muddled pile on the table. People who have built an aircraft as complicated as the F17 probably know in their bones that nothing but the best will work, and that the best will cost a lot.

The counsel's intermediate case is not hugely technical. These are early days, when no single way of attacking and destroying presumably hostile missiles can be singled out. For the time being, keep all options open. But lasers have the disadvantage of being inefficient in their use of energy, charged particle beams are necessarily so distorted by the Earth's magnetic field that their power density at a distance will be enormously decreased. Beams of neutral particles, say hydrogen atoms, look a much better bet.

But will not any such space-based weapon be vulnerable to counter-measures? Not necessarily. Since any attacking projectile will have to follow a ballistic trajectory, that too will be as visible as the satellites in which the SDI is embodied, and will not prudently be equipped with other than short-range mechanisms of destruction. So why not equip the satellites to evade whatever is sent to get them?

By these standards, the arguments there have been about the number of the satellite-based battle stations that would be necessary to destroy a hostile force of missiles appear to be mundane. Both the congressional Office of Technology Assessment and the Union of Concerned

Scientists are probably right to have argued that the number required may be larger than the 180 or so that the Pentagon first calculated, but the Pentagon is probably right to argue, as it does implicitly, that only the first will cost really big money.

The temptation to base the case against star wars on the argument that its components will not function individually is probably mistaken. Enough people in the technical community seem in the past few months to have been persuaded that there is something in the scheme to give the lie to that. But it seems also to be agreed that the most formidable and still unsurmounted problem is that of handling the data from all those radars, and for deciding automatically, in minutes, how to respond.

The third line of defence against criticism takes two forms. First, because there are five phases during which hostile missiles can be attacked, with the first (soon after launch) and the last (when the decoys have been slowed by atmospheric resistance) most easily recognizable, so each single stage of the defensive system does not have to be perfect. Indeed, the defenders' advantage is even greater, because a hostile missile force would presumably have been assigned targets that must necessarily be destroyed, and will be bound to counter even imperfect defence by duplicating its chosen targets.

The second and newer defence is a continuation of that theme: the initiative is really a way of forcing an adversary to recognize that there is no alternative to arms control. Provided that the efficiency of the defensive system is more than nearly zero, there will be limits to the extent to which it can realistically be countered by building more strategic missiles. But how will an adversary know how effective the nascent system will be? For that matter, how will the designers know the performance of a system they cannot test?

There is necessarily a sense of unreality at this point. The most common complaints against SDI, that it cannot work, seem to outsiders to be belied by the numbers of intelligent people who are passionately persuaded otherwise. (But everybody admits that the cost could be unaffordable, or is at least a problem.) The strongest arguments against are strategic and political. But even there there is a defence; whatever happens to SDI's research phase, only the next US administration will have to decide what should be done.

John Maddox

Astronomy

What is at the centre of Halley's comet and how fast does it spin?

from David W. Hughes

THE source of all the gas and dust emitted by Halley's comet as it speeds past the Sun is thought to be a compact, dirty snowball nucleus. Compact because the comet's mass of 2×10^{17} g is confined in an object of mean diameter 9.4 km. Dirty because around 25 percent of the mass is made up of silicate particles weighing between 10^{-15} and 10 g, with a composition similar to carbonaceous chondrite meteorites, and probably evenly mixed with what is predominantly H_2O snow. At its last apparition in 1910, the comet lost about 2×10^{14} g of material, equivalent to peeling a layer 200 cm thick from the surface of the nucleus. Observations of Halley's comet over the past 2,000 years are consistent with the same rate of surface loss at each apparition. But what does the nucleus look like? And how fast is it spinning?

At this approach to the Sun, the comet was first seen on 16 October 1982 when it was 11.04 AU away. It is in opposition (on the opposite side of the Earth to the Sun) around January and during that time observers have been carefully monitoring its brightness. Two papers detailing last year's results have been published in a recent edition of *Astronomy and Astrophysics*. One group (Le Fevre, O. *et al.* *Astron. Astrophys.* **138**, L1; 1984) used the 3.6 m Canada-France-Hawaii telescope. The other (West, R.M. & Pedersen, H. *Astron. Astrophys.* **138**, L9; 1984) used the 1.5 m European Southern Observatory telescope at La Silla, Chile. Both found that the brightness of the comet is changing not only because of the slow variation in heliocentric and geocentric distances but also throughout the night and from night to night.

An analysis of the comet's absolute magnitude (magnitude reduced to the idealized position where the comet is 1 AU from both the Sun and Earth and seen at full phase) shows that this quantity did not change perceptibly between October 1982 and March 1984. During this time the heliocentric distance of the comet decreased from 11.05 AU to 7.75 AU, and the insolation went up by a factor of two. So it is reasonable to suppose that the cometary snow was not sublimating, dust was not being accelerated away from the nucleus and the comet was inactive. This conclusion is supported by the fact that the comet image was perfectly round and very similar to the telescope's seeing disc.

Magnitude can be measured to ± 0.2 . West and Pedersen found that the night-to-night variation had an amplitude of 1 magnitude (equivalent to a brightness

change of 2.5), and Le Fevre *et al.* found an amplitude of about 1.7 magnitudes (equivalent to a brightness change of 4.8). Two explanations, both relying on the spin of the nucleus, have been put forward to explain this variation.

The first assumes that the surface of the nucleus has a uniform albedo and that the nucleus is not spherical but elongated. A lemon-shaped object, spinning about its axis of maximum moment of inertia (this axis being perpendicular to the plane of its orbit), would give the observed brightness readings if the long axis was about 1.9 times larger than the short axis. The brightness is simply proportional to the area of cross section presented to the observer. One problem with this hypothesis is that asymmetrical outgassing when the nucleus is close to perihelion would induce a precession of the spin axis, whereas orbital analysis indicates that the two non-gravitational parameters have remained reasonably constant over the past 2,000 years showing that the precession rate must be low.

The second possibility is that the nucleus is nearly spherical but the reflectivity varies over the surface. Maybe it is like Saturn's satellite, Iapetus. This has a synchronous spin period (always presenting the same face to Saturn) but, as it orbits the planet the leading face is dark (albedo ~ 0.045), whereas the trailing face is much brighter (albedo 0.5). The spin axis is near the plane of separation of the two hemispheres. Notice that the brightness of the nucleus would be proportional to the mean albedo of the surface facing the observer. One hemisphere could be covered with friable carbonaceous dust with a lunar-like albedo of 0.07, the other could be characterized by exposed areas of ice with an albedo of

0.6. But how is such an asymmetrical and inhomogeneous nucleus produced? And how could this lack of symmetry persist after many millions of rotations and the 2,300 or so close solar passages that Halley's comet has already endured? There again, complicated jet and fan-like structures have been seen in the inner comas of comets. These could arise from active zones on the rotating nucleus producing a burst of gas and dust as they are heated by being spun through the subsolar point.

Returning to Halley's comet, what is the spin period of the nucleus? Whipple (*IAU Circular* 3459, 13 March 1980) analysed photographs and drawings of the 1910 and 1835 apparitions, paying special attention to the evolution of selected dust-ejection features, and tentatively set a 10.3 h spin period. Sekanina and Larson (*Astron. J.* in the press) returned to the problem and concluded that the period was about 41.5 h. LeFevre *et al.* suggest that their data can be fitted by a period of 48–51 h but stress that, if the brightness variation is sinusoidal, a period of 24.3 ± 0.3 h is also very likely and that submultiples (of approximately 12.2 h and 8.1 h) may also agree with the observations.

This confusion is not helped by the fact that an observer based at a single observatory on the spinning Earth can only see the comet for about 3 hours out of 24. Has the problem been solved by a collusion of observers in Chile, South Africa and Australia? By December 1984, the comet was within 5.5 AU of the Sun, the snow was starting to sublimate and the cometary nucleus had become shrouded from view by the brighter surrounding coma. If the relevant data have not been collected, the measurement of the spin period of the nucleus will have to wait for the Giotto spacecraft to fly by the comet in March 1986 or until the coma has dissipated as the comet moves away from the Sun into the deep freeze at the boundaries of the solar system. $\square$

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Neuroscience

Skeleton key to memory?

from N. R. Burns

COUNTERPARTS to the proteins that form the skeleton on the inner surface of the membrane of the red cell have been found in many other cell types (see ref. 1), in which they are presumed to stabilize the membrane. But searches have also been made for a more dynamic function of the membrane skeleton, for example in the transduction of signals from plasma membrane receptors to the interior of the cell. The latest and most dramatic claim, however, which emanates from G. Lynch and

his co-workers, is that the skeleton has a function in the mechanism of memory.

When certain synapses in the rodent hippocampus are subjected to brief, high-frequency stimulation, the postsynaptic potentials evoked by subsequent stimulation are significantly increased. This is referred to as long term potentiation (LTP). It may last for days and has been considered a possible model for the basic synaptic changes that underlie learning and memory. Lynch and his colleagues have

presented evidence that one accompaniment of LTP is an increase in the number of receptors for glutamate, which is the putative neurotransmitter at these synapses.

Last year Lynch and Baudry² unveiled a theory, in which fodrin (a neuronal analogue of spectrin, the principal protein of the red cell cytoskeleton) plays an essential part in these changes. They suggest that, in the neurones stimulated in LTP, there is an increase in postsynaptic calcium concentration; this then activates the calcium-dependent protease, calpain 1, which uniquely attacks fodrin. The degradation of fodrin leads to exposure of cryptic postsynaptic glutamate receptors, so increasing the postsynaptic potentials evoked by subsequent exposure to glutamate.

In support of the theory, Siman, Baudry and Lynch³ have demonstrated a relationship between the calcium-stimulated enhancement of glutamate binding and the degradation of fodrin, both effects being blocked by leupeptin, an inhibitor of calpain 1. More remarkably, perfusion of rats' brains with leupeptin, which on Lynch's scheme should inhibit their capacity to learn, indeed impairs the performance of various behavioural tasks. On page 225 of this issue, the same authors provide evidence that causally links the degradation of fodrin to the calcium-stimulated increase in glutamate binding⁴.

Siman *et al.* have used antibodies (or their univalent fragments) to fodrin to protect the fodrin on hippocampal synaptic plasma membranes against calpain 1. The membranes thus treated no longer increase their glutamate binding in response to calcium; a good correlation is observed between the amount of antibody bound and the response. This demonstrates that the effect of leupeptin is not a consequence of blocking calpain activity *per se*. It is also shown that the antibody effect does not arise from any sequestration of calcium or from a non-specific perturbation of the membrane by immunoglobulin. In the absence of antibodies only some 20 per cent of the fodrin is degraded, suggesting that a sub-population, possibly localized, is involved in the effect. (It is perhaps worth recalling that when similarly activated by calcium in the red cell, calpain 1 does not preferentially attack spectrin, but rather its associated proteins, 4.1 and ankyrin⁵, which coexist with fodrin in the neurone).

The sequence of events posited by Siman *et al.* certainly embodies important features required for a physiological mechanism of memory. The initial event could be effected by the brief physiological changes that accompany synaptic transmission. The scheme also accommodates the requirement for a long-lasting change, for if the membrane cytoskeleton regulates the shape of the neurone, as it does that of the red cell, then a structural perturbation could readily be envisaged to result in the altered ultrastructure of the dendritic spines that seems to occur in the hippocampus of rats subjected to LTP⁶.

The elucidation of the molecular basis of memory has been frustrated by the extremely circumscribed nature of the elicited response, which is beyond existing techniques to discriminate. Although significant progress has been made in understanding changes in synaptic efficacy in the nervous system of *Aplysia* and other invertebrates⁷, our knowledge of the repertoire of possible presynaptic and postsynaptic changes is still fragmentary. If, indeed, something as simple as the cleavage of a single protein represents a major and uni-

versal event in the process of learning, this would be cause for new hope, if not yet for dancing in the quad. □

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Atmospheric physics

Atomic oxygen from a balloon

from Gaston Kockarts

REMOTE sensing techniques provide an efficient way to investigate the gas composition and structure of the terrestrial atmosphere. It is, however, necessary to avoid the problems of absorption by other atmospheric constituents and to have an instrument with a sufficiently high resolution to discriminate the spectral signature of a specific component from others. Although the infrared emission at 63 μm of atomic oxygen has been detected a few times during rocket flights above 85 km altitude in the thermosphere, T.A. Clark and colleagues have achieved the first measurements at stratospheric height, by using especially developed detectors carried in a balloon. Their measurements, reported on page 206 of this issue, are important because this emission is thought to play a significant role in the cooling of the upper atmosphere.

A physical nomenclature of the Earth's atmosphere can be based on the vertical distribution of temperature. Starting at ground level, the troposphere is characterized by a decrease of temperature, which increases again in the stratosphere, above 10 km, owing to the absorption of solar ultraviolet light by ozone. Above 50 km, in the mesosphere, the temperature decreases again as a consequence of infrared cooling by carbon dioxide. It reaches a minimum of 150–200 K at approximately 85 km, where the thermosphere starts. In this region, atmospheric molecular oxygen is progressively photodissociated by solar ultraviolet radiation of a wavelength shorter than 175 nm; in this way, atomic oxygen becomes a major atmospheric constituent. The absorption of solar ultraviolet radiation induces a heating of the thermosphere, the temperature of which increases with height until it reaches an altitude-independent value above 500 km of 600–1,800 K depending on the level of solar activity. Heat is transported downwards by thermal conduction, but can also be removed from the atmosphere by infrared emission from atmospheric constituents which have been excited through collisions or through reactions with other components.

The spectroscopic ground state of atomic oxygen is a triplet level and the excitation energy for the 63 μm line is only 0.02 eV, which is of the same order of magnitude as the thermal energy of the thermospheric constituents. Therefore, by collisions with the ambient gas, atomic oxygen in its ground state can be excited and the 63 μm line emitted. For a long time, this mechanism was considered the principal cooling effect in the thermosphere. However, in *Geophysics Research Letters* 7, 137, 1980, I showed that the 5.3 μm infrared emission from nitric oxide, a minor thermospheric constituent, is more efficient; paradoxically, the fundamental reason for less efficient cooling by atomic oxygen is its great abundance, which is responsible for a reabsorption of the 63 μm emission. The complexity of the radiative transfer problem will be further increased if the usual assumption of local thermodynamic equilibrium is not valid, which can only be ascertained by accurate measurements of the 63 μm emission.

Clark and colleagues demonstrate the feasibility of observing the infrared emission of atomic oxygen from stratospheric heights. The interferograms obtained with the Fourier spectrometers have sufficient resolution and sensitivity to discriminate the 63 μm emission from atmospheric emissions such as water vapour and its isotopes. Were the technique to be adapted for use on a satellite, it could lead to vertical profiles of the infrared emission of atomic oxygen, and, in principle, such profiles could be used to determine the abundance of atomic oxygen, which is the progenitor of atmospheric ozone. Although fundamental atmospheric infrared observations are usually made at shorter wavelengths, Clark *et al.* have shown that an astronomical detector, such as theirs, can provide valuable atmospheric results in the 50–100 μm wavelength range, showing the value of a multidisciplinary approach. □

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Behavioural ecology

Sex among the dunnocks

from Paul H. Harvey and David S. Wilcove

ON FIRST encounter, the dunnock (*Prunella modularis*) seems a most uninteresting small brown bird. Yet, within a single population, this species can show astonishing diversity in its breeding behaviour. Working in Cambridge University's botanic garden, Nick Davies and Arne Lundberg have found breeding groups of dunnocks that are polygynous (several females, one male) polyandrous (several males, one female), polygynandrous (several females, several males) and monogamous. This variation has allowed them to test, in a specific case, the hypothesis that female vertebrates often distribute themselves according to the spatial distribution of resources, while males respond to the distribution of females. The result is published in a recent issue of the *Journal of Animal Ecology*¹.

Between January and March dunnock males set up singing territories which they vigorously defend against other males. In the spring, the females also begin to form territories which they defend against other females. The females' territorial boundaries are not constrained by those of the males, but the overlap of the territories of the two sexes seems to determine the diversity of mating systems. Some females range entirely within the territory of a single male and the two birds form a monogamous pair. Occasionally, the range of a female encompasses the contiguous territories of two males and this results in polyandry. Initially the males squabble and each wins the encounters that occur on its own turf, but one male gradually emerges as the dominant member of the pair, their territories coalesce and the dominant male subsequently copulates with the female more often than does its rival. When the ranges of several females are small enough to be contained within the territory of a single male, the result is polygyny. The final type of mating system, polygynandry, occurs when two or more male territories fuse to encompass the ranges of two or more females. In the polyandrous and polygynandrous systems, where more than one male mates with a single female, the males do not seem to be related, unlike in most such avian breeding systems.

Clearly, the dunnock's breeding system is influenced in large part by the size of female territories. What, then, determines female territory size? The answer is food. Dunnocks feed on small seeds and invertebrates which they glean from the ground in patches of dense vegetation. Within the botanic gardens of Cambridge University, the size of a female dunnock's territory is inversely related to the local density of such patches. Davies and Lundberg were able to manipulate female territory sizes by altering

the food supplies of some birds and demonstrated a consequent change in the breeding system of individual dunnocks. Thus, supplementing the food supply results in smaller female territories that are more often monopolized by males. This shifts the mating system away from polyandry; the proportions of unpaired males and polyandrous associations decline, while monogamous pairs and polygynandrous associations increase sharply.

Other experiments have successfully test-

ed the resource dispersion hypothesis^{2,3}, but not in a species with such a diversity of mating systems as the dunnock. Davies and Lundberg's study is one of a growing number to show that many bird species have quite diverse sexual habits. It also provides an example of a species that is not restricted to only one or two mating systems by psychological, life history or phylogenetic constraints, contrary to the conclusions of a recent review⁴. □

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Solitons

Optic fibres, water tanks and the hydrolysis of ATP

from John Elgin

A SOLITON is a rather special type of large-amplitude solitary wave, the integrity of which is maintained by a balance between nonlinearity and dispersion within the medium through which it propagates. Although observed well over a century ago in connection with water waves, the ubiquity of the soliton has emerged only in the past twenty years or so, and the stable nonlinear excitation that is the soliton has now been observed in many physical systems. Three new developments in the theory and application of solitons were of particular interest at a recent conference*.

L.F. Mollenauer (Bell Laboratories, Holmdel) offered one of the more exciting applications of soliton physics. A soliton laser is used to produce pulses of 210 fs duration at a wavelength of about 1.5 μm . Simple extensions to the basic experimental scheme could reduce the width to a mere 25 fs (about 5 optical cycles). The basic experimental scheme (Mollenauer, L.F. & Stolen, R.H. *Opt. Lett.* **9**, 13; 1984) is a synchronously-pumped colour-centre laser (homogeneously-broadened) that is tunable from 1.4 μm to 1.6 μm and produces output pulses typically of about 8 ps full width at half maximum. To operate as a soliton laser, the output pulses from the laser are first inserted into a length L of single mode optical fibre and then reinjected back into the laser cavity for further amplification. The optical fibre introduces the 'soliton' aspect into the system. It is well known that the appropriate equation governing the evolution of the field E , for optical pulses propagating through a fibre, is the nonlinear Schrödinger equation, where the nonlinearity arises from the refractive index

($n = n_0 + n_2 |E|^2$). This equation has both single- and multi-soliton solutions. In particular, for the double-soliton solution, the input pulse becomes two solitons, both of which propagate with the same velocity, and which 'beat' against each other somewhat in the manner of two tuning forks. The result is that the pulse profile within the fibre is (spatially) periodically modulated, becoming extremely narrow at the half-period point. When reinjected back into the laser cavity, these narrow pulses force the laser to produce still narrower pulses, and so the cycle repeats. The periodicity length L' scales as the square of the input pulse duration, and hence decreases as the pulses narrow. When L' is equal to the fibre length L , a stationary state results. Mollenauer presented recent experimental results that clearly demonstrate these effects, but he indicated that a proper theoretical treatment of the problem is outstanding. More generally, the work has useful consequences for information technology systems based on optical fibres.

The role played by solitons in biological systems was discussed by A.C. Scott (Los Alamos). They have been implicated by A.S. Davydov (*Phys. Ser.* **20**, 387; 1979) because of the following problem. The hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate releases about 0.42 eV of free energy. A basic biological resonance is the double-bonded carbon-oxygen (or amide-I bond) which has a quantum energy of 0.205 eV (1,650 cm^{-1}), and is found in every peptide group of every protein. It is tempting to postulate that the amide-I bond takes up the energy released during ATP hydrolysis (in either single or double quanta) and stores or transports it along the polypeptide

* 'New Developments in the Theory and Application of Solitons', Royal Society, London, 1-2 November 1984.

chain. However, typical amide-I absorption peaks imply a lifetime of about 1 ps, which is too short for normal biological mechanisms. Davydov suggested that this lifetime could be markedly increased if the excitation interacted nonlinearly with other excitations on the polypeptide chain. In particular, the localized amide-I bond energy could act as a source for phonon (sound) waves excited along the chain, which in turn could act as a potential well to trap the bond energy and prevent its dispersion. The complete excitation would therefore be localized and inherently nonlinear in nature, that is it would be a soliton. Scott and his colleagues have spent several years studying and extending Davydov's model. Recently, they have used it to interpret spectral lines measured in Laser-Raman studies of metabolically active *Escherichia coli* (Scott, A.C. *Phys. Rev. A* 26, 578; 1982), arguing that such spectra are consistent with the notion of soliton excitation along the chain. Scott also presented evidence of self-trapping of amide-I vibrational energy in crystalline acetanilide, which can be similarly explained in terms of the Davydov soliton excitation hypothesis.

Returning us to the original hunting ground for solitons—fluid mechanics—J.B. Keller (Stanford University) described the way in which solitons are generated and propagate in terms of initial value and initial boundary-value problems. Experiments in which a ship is pulled along a narrow test tank demonstrate soliton excitation. A critical parameter F_R (ship speed/speed of long-wavelength, small-amplitude waves) exists, such that solitons are excited only when $F_R > 1$. Because of the walls of the test tank, the solitons propagate ahead of the ship—hence the need to model the system as an initial boundary-value problem rather than simply as an initial value problem in studies on the soliton properties of the Korteweg de Vries equation, for example.

Seven other papers reflected the rich variety of topics to which solitons have relevance and also dealt with the more abstract and mathematical topics of algebraic and topological structure of the soliton equations themselves. □

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High-pressure physics

Doubly-shocked helium

from P.V.E. McClintock

IN EXPERIMENTS carried out by W.J. Nellis and colleagues at Lawrence Livermore National Laboratory in California, and reported in *Physical Review Letters* 53, 1248; 1984, liquid helium has been shock-compressed to densities of up to five times that of the normal liquid. The pressure transiently achieved in the experiments approaches that to which helium is subjected on some planets.

The behaviour of matter at extreme densities is of particular interest not only for its own sake but also because of its relevance to an understanding of the giant planets. Saturn, for example, appears to have an internal energy source of some kind and it is possible that the unmixing and gravitational separation of the hydrogen-helium fluid is responsible for the evolution of heat. Whether such phase separation occurs in reality is not at all easy to resolve by laboratory experiments because of the extreme pressures and temperatures on Saturn, which are believed to range up to about 1 terrapascal (10^7 atmospheres) and 14,000 K respectively. To approach the requisite pressure a number of powerful compression methods are being developed. These include static compression techniques, the controlled use of explosives and the operation of a two-stage light-gas gun, as used by Nellis *et al.*

The light-gas gun, which can be considered as a quite monstrous analogue of an airgun, is used to fire a flat-ended projectile

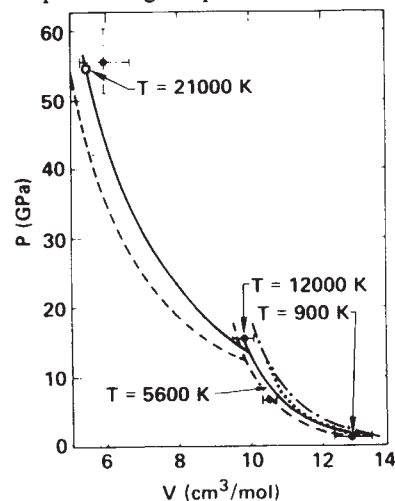
at very high velocity on to the sample under study, which is thereby transiently compressed. To maximize the initial, and thus also the final, densities the sample is usually chosen to be in its solid or—as in the present case—liquid phase. The process is initiated by the firing of a charge of gunpowder in a combustion chamber. That drives a piston up a gun barrel of relatively large diameter, and the piston very rapidly compresses hydrogen gas into a second and much narrower barrel, along which the projectile is propelled at a large and approximately constant acceleration, finally emerging from the muzzle at a velocity of between 3 and 7 km s⁻¹. When the projectile strikes the outside of the sample holder, a shock wave is generated and propagates through into the liquid helium, carrying with it a region of extreme pressure. As a result, there are almost instantaneous and dramatic increases in the temperature and pressure of the helium, which are further increased when the shock wave reflects from the rear surface of the sample holder.

Notwithstanding the extreme (and destructive) speed with which these events occur, it is still possible to acquire detailed information about the state of the sample during the shock. The velocity of the projectile before impact is determined precisely by means of a flash X-ray technique, and the velocity of the shock waves is measured from their transit times between self-

shorting coaxial metal pins (relying on the fact that many materials that are normally insulators become conductors under conditions of extreme pressure). From a knowledge of the initial density of the liquid helium and the characteristics of the projectile and sample chamber it is possible to calculate the shock pressure, density and temperature by use of the so-called Rankine-Hugoniot equations. By such means, Nellis *et al.* calculate that the first shock transiently increases the temperature of the helium from 4.3 K to 12,000 K and its pressure from just above atmospheric to 16 gigapascals. After reflection of the shock wave, the 'double shock' takes the temperature to 21,000 K and the pressure to 56 gigapascals.

The results are compared with some theoretical predictions in the figure. Each of the broken curves is derived from a different helium interatomic potential based on past work. Only the best of these (dashed curve) has been extended to pressures above that of the first shock, since it is immediately evident that the others are in serious disagreement with experiment. The full curve represents an improved version of the theory shown by the dashed curve, adjusted in the light of the single-shock results; and the open circle represents the resultant prediction of where the double-shock data point ought to fall. It is, in fact, in excellent agreement with prediction, which can be regarded as reassuring evidence in support of the self-consistency of the data analysis and the accuracy of the adjusted interatomic potential.

Although the pressures achieved are still not up to the highest pressures found in the



Comparison of shock-compression results for helium (points with error bars) with theoretical predictions (curves), the pressure P being plotted as a function of molar volume V . From Nellis, W.J. *et al. Phys. Rev. Letters* 53, 1248; 1984.

giant planets, they are getting remarkably close, so laying a firm foundation of experimental data for the building of theories to describe those distant worlds. □

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Oceanography

Out of the SWAMP to a more certain life on the ocean wave

from Robert Bryan Long

WEATHER forecasting and analysis could be considerably improved by the availability of numerical models that are good enough to define the coupling between meteorology and the waves on the surface of the ocean. Spurred on by this need, by the impending appearance of a new series of satellites that can sense ocean waves, and by the findings of the improbably named SWAMP group, numerical modellers from nine countries and representing 23 institutions have recently formed a coalition in order to develop a third generation of surface-wave models.

Simulation of the behaviour of the ocean surface under the influence of wind has seen a growing number of applications in recent years. Since 1974, the US Navy has run a northern hemisphere Spectral Ocean Wave Model (SOWM)¹ that provides forecasts for ship routing and other naval activities. A global version of the same system is now being implemented. In Europe, the British Meteorological Office has a regional model (designated BMO)² in routine use for the North Sea and the adjacent Atlantic, while in the Netherlands, the GONO model³, which covers the North Sea and the Norwegian Sea, is run four times a day and provides valuable information in support of the massive Dutch coastal engineering projects. In Norway, a

new model developed in the United States has been adopted to provide wave forecasts for the oil industry in the Norwegian Sea. And another dozen or so numerical wave models, which are in advanced stages of development or already in operation, are used either to generate forecasts of ocean wave fields or to produce engineering statistics by hindcasting wave fields for statistically adequate samples of storms.

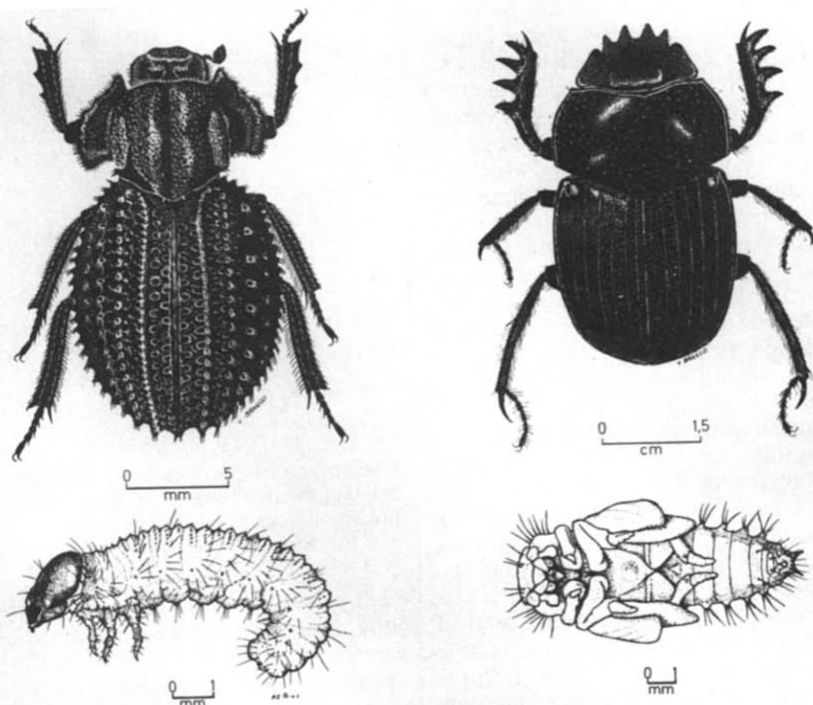
Several years ago, wave modellers from the United States, Europe and Japan, calling themselves the SWAMP group (Sea Wave Modelling Project), subjected the models from the institutes they represented to a sequence of six different wind-field geometries, each designed to explore a separate aspect of wave prediction (such as fetch-limited and duration-limited growths, the effects of asymmetrical fetch geometries, inhomogeneous and/or non-stationary wind fields, swell generation and propagation). A seventh test used wind fields that simulate stationary and moving hurricanes. The results of that study, soon to be published by Plenum Press in a book tentatively entitled *Ocean Wave Modelling*, indicate that the state of the art is somewhat less than satisfactory.

All current numerical models of wave-prediction are based on computer-generated solutions to a radiative transfer

equation describing the balance of processes controlling the evolution of the surface-wave number spectrum. These processes include advection, refraction and a variety of sources and sinks of energy. The principal energy source is the wind, while the principal sink is dissipation due to wave breaking and, in shallow water, to interaction with the sea bed. In addition, the non-linear interaction between waves acts as either a source or a sink for a given wave number. The advection, refraction, and non-linear processes are fully understood from first principles but the atmospheric input and dissipation effects are not well understood and must be modelled empirically. Existing wave models differ according to the set of parameters onto which the radiation balance is projected before being presented to the computer for solution, the particular mix of theory and empiricism used to model the source/sink terms, and in the details of the numerical techniques employed.

The SWAMP group classified existing numerical wave models as first or second generation according to their treatment of linear effects. First generation models, of which SOWM is an example, omit the non-linear term in the radiation balance (or model it in such a way that its effect is negligible), leading to an essentially linear formulation in which each wave component independently grows, propagates, saturates and decays. The prototypes of these models were designed before the importance of the weak non-linear interaction between waves was established by the theoretical work of Hasselmann⁴ and experiments of the Joint North Sea Wave Project (JONSWAP)⁵. Second generation models incorporate the non-linear interaction in some form. In principle, the non-linear effects can be exactly computed, but the calculation is so time consuming that it has, until recently, seemed impossible to make it an integral part of a full, three-dimensional (2-space and time) wave model. (One of the SWAMP models, optimistically called EXACT-NL, explicitly computes the non-linear interaction but only for one-dimensional problems, such as simple fetch-limited or duration-limited growth.)

Although there are wide variations in the parameters used to incorporate non-linear interactions into second generation models in the SWAMP study, in every case they include some kind of external constraints on the permitted shapes of the wind-sea portion of the spectrum, where the non-linear transfer is known to be important. These constraints force the wind-sea spectra



A selection of the scarabid dung beetles of South Africa described by A.J. Prins in the *Annals of the South African Museum* 94, 203; 1984. Top left, *Trox horridus*; top right: *Nenteuchus proboscideus*; bottom, larva and pupa of *Trox rhyaroides*.

Correction

In the article "Draughtsmen of the constellations" by David W. Hughes (*Nature* 20/27 December, p.697), 0^0 on line 10 should have been $90 - \theta^0$, 0^0 on lines 13 and 64 (twice) should have been θ^0 , longitude on line 25 should have been latitude, each $\frac{1}{2}$ should have been $\pm$, and $\sim$ was omitted before 125BC on line 19.

to conform to empirical rules established largely on the basis of JONSWAP data. On the other hand, swell components (defined in this context as long waves, well removed from the peak of the wind-sea spectrum) are essentially uncoupled from the wind-sea and must be individually considered as linear free waves. Differences in the definition of the transition from the wind-sea to swell, and in the representation of the non-linear interactions probably explain why agreement between second generation models in the SWAMP study is no better in quality than that between the two generations.

So there are two principal objectives in the new joint initiative to develop third generation models. First, all wave components in the spectrum, whether swell or wind-sea, must be treated in the same way and allowed to evolve freely. Second, the Boltzmann integral quantifying the non-linear interaction needs to be evaluated at every grid point and time step, if not exactly, at least more accurately than in the second generation models. Already, participants from the Max Planck Institute for Meteorology in Hamburg, FRG, have

run a prototype global, deep-water third generation model on the Cray computer at the European Centre for Medium Range Weather Forecasts with results which, though very preliminary, are promising. The development of regional versions of the global model is planned, including finite-depth effects where necessary. A programme of verification studies, using wind and wave data collected by the participating institutes, is also planned. The project, presently being coordinated by G. Komen of the Royal Netherlands Meteorological Institute, is a commendable example of international cooperation in pursuit of a common goal, and offers some exciting prospects. □

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Bacterial motion

Square is beautiful

from Michael Spencer

NOT many things in the natural world are square. Hexagonal objects are common enough; but on the whole it is the smooth curves of circle, ellipse and spiral that dominate the evolutionary drawing board. In human affairs, this preference for the well-rounded extends from beauty contests to the marketing of food. Some years ago, the British Egg Marketing Board devised an ingenious solution to the problem of packaging millions of fragile, odd-shaped objects: the eggs would be fed into a vast machine to be individually broken and resealed in square-sided plastic boxes. No more was heard of the scheme, doubtless due to market research indicating that the customer would not stand for it. The same aesthetic sense must have dictated that potato crisps (US chips), now made by cutting up sheets of processed potato paste, still retain the pleasing contours of a thinly-sliced tuber.

Biology, however, offers an example of almost any shape if you look hard enough. This august journal achieved some kind of

scoop a few years ago by publishing the first sighting of square bacteria (*Nature* **283**, 69; 1980). These surprising objects are to be found lurking in the briny pools of southern Sinai. A new report by M. Alam *et al.* (*EMBO J.* **3**, 2899; 1984) concentrates on their motile behaviour. This is of unusual interest because of the discovery that at least one kind of halobacterium (*H. halobium*) swims differently to other bugs: it goes backwards and forwards by simply reversing the sense of rotation of its right-handed helical flagella, rather than going through the more normal cycle involving a change of the helical sense. As I have recently pointed out in these columns (**310**, 367; 1984), this presents a conundrum to those trying to understand how individual flagellar motors could operate; it requires a rather tortuous argument (or an entirely new model) to explain how a bundle of many flagella could reverse its direction of rotation without getting tangled up.

Alam *et al.* find that square bacteria, which have apparently not yet been graced

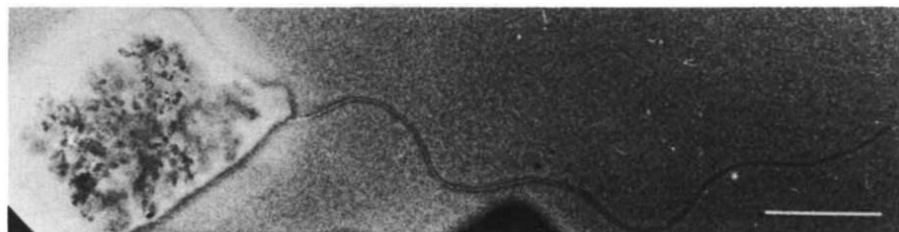


100 years ago

AT a recent meeting of the German Asiatic Society of Japan a paper was read by Dr. H. Muraoka of Tokio, on the magic mirror of Japan. It is generally supposed that its magical quality was discovered only recently; but it was, says Dr. Muraoka, known for a long time in Japan. Old ladies have told him that in their youth, fifty years since, they frequently noticed, when at toilet, that the reflection of the sun from the mirror on the wall or ceiling contained the figures or letters on its back. It is said to have been known to the Romans in connection with some of their own mirrors, and any one concealing a mirror possessing this quality was arrested as a sorcerer; but the authority for this statement is not given. The subject is engaging considerable attention, as will be seen from the fact that in recent years a list of fourteen writers on the subject is quoted, from Stanislas Julien, in 1847, to Messrs. Ayrton and Perry quite lately; and, as the subsequent discussion showed, there are omissions even in this list. These writers, especially the latter, have demonstrated beyond doubt that unequal convexities in the mirror beget its magical quality. The polished surfaces are convex, but the convexity is not continuous, and is broken in certain places. After going over what had already been done on the subject, and its results, the author described his own investigations. The riddles of the mirror are far from being all answered by the discovery of unequal convexity. For example, how is the inequality caused—by pressure, heat, or by changes in the molecular tension of the metal plates? The writer tried many experiments to answer the question, and he succeeded by means of chemical agents in drawing lines on the flat back of a mirror, which were reproduced on a reflected image from the front.

From *Nature* 31 249, 15 January 1885.

with a taxonomic title (*H. quadratum*? *H. incredible*?), pose the same problem as their better-known cousins. Their flagella are right-handed helices; and as they swim about, for all the world like animated postage stamps, they reverse direction by simply changing from clockwise to anti-clockwise rotation. Although the flagella show a strong tendency to stick together in bundles, reversal does not cause the bundles to fly apart as it does in non-halophiles. One of the possible let-outs suggested by earlier work now seems less plausible because electron micrographs show that large bundles or 'super-flagella' are unlikely to be detached flagella aggregating around a single rotating filament. It seems they really can be formed by filaments arising at different points on the cell surface. Even more oddly, the slower contrary rotation of the cell body is sometimes absent. Rather teasingly, Alam *et al.* remark that this interesting problem will be dealt with in another communication. This one should run and run. □



Electron micrograph of a square bacterium with a single flagellum negatively stained with phosphotungstic acid. The bar indicates 1 μ m. From Alam, M. *et al.* *EMBO J.* **3**, 2899; 1984.

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Epidermal growth factor

Is the precursor a receptor?

from Suzanne Pfeffer and Axel Ullrich

THE recent elucidation of the sequences of the cell-surface receptors for low density lipoprotein (LDL)^{1,2} and epidermal growth factor (EGF)³ have revealed an unexpected relationship between their structure and that of the biosynthetic precursor for EGF itself^{4,5}, and have enhanced speculation that the EGF precursor may play a dual role as both a growth factor precursor and a cell-surface receptor. New clues to this puzzle are provided by Rall and co-workers⁶ on page 228 of this issue of *Nature*.

The 53-amino acid, mature EGF molecule is encoded within a 1,217-amino acid EGF precursor (refs 4* and 5). It contains an internal stretch of 20 hydrophobic amino acids, flanked by polar residues, which could anchor the precursor in the membrane and would divide it into a 159-amino acid cytoplasmic domain and a 1,010-amino acid extracellular domain (see figure). Why would a secreted growth factor be encoded within a membrane-bound precursor, and why is the precursor more than 20 times larger than the mature growth factor? In addition to EGF coding sequences, the precursor contains eight repeated units of a sequence that is related to EGF. Each unit encodes approximately 40 amino acids including 6 cysteine residues. These units may represent other biologically active polypeptides but, unlike the polypeptide units in precursors such as pro-opiomelanocortin⁷, they are not flanked by obvious proteolytic cleavage sites.

Striking amino acid sequence similarities have recently been detected between bovine¹ and human LDL receptors² and the mouse EGF precursor. The most significant similarity (about 40 per cent) is between EGF precursor amino acid residues 565-701 and residues 457-595 in the LDL receptor extracellular domain (see figure). Sequence homology decreases in regions surrounding this core, but remains statistically significantly (approximately 30 per cent) for several hundred more amino acids. In addition to direct sequence homology, the LDL receptor and the EGF precursor share other structural features. Like the EGF precursor, the extracellular domain of the LDL receptor contains eight repeated, cysteine-rich units of 40 amino acids in length. The spatial distribution of the cysteine residues is conserved in each of the repeat units in both molecules but the relative positions of the cysteines differ between the LDL receptor and the EGF precursor repeat units. In both cases, other amino acid residues seem also to have been conserved. The repeat units of both proteins are distributed into two larger, distin-

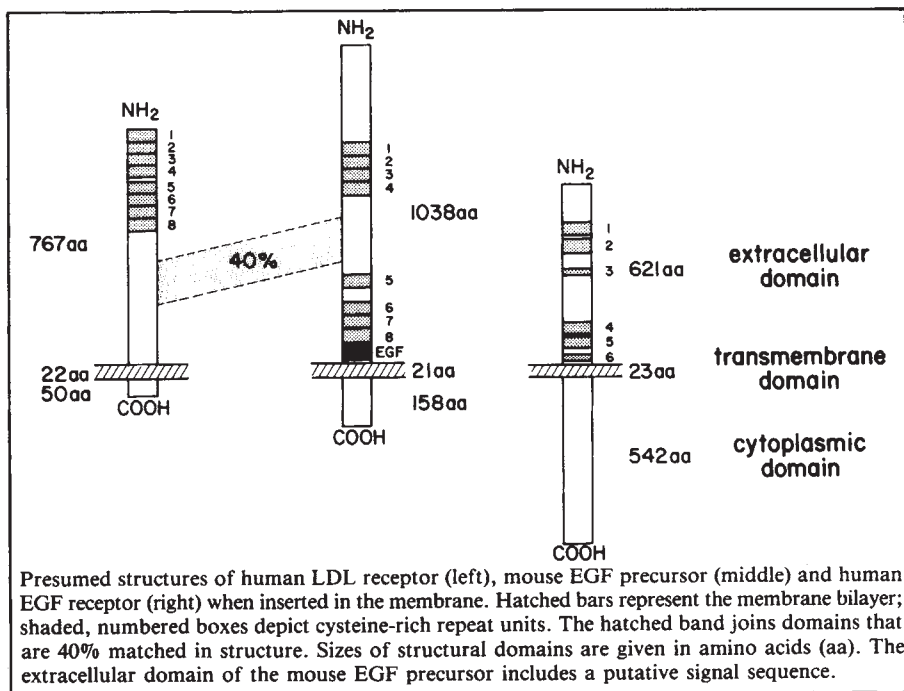
guishable units, which are of similar size, and are arranged in tandem in the LDL receptor, yet are separated in the EGF precursor (see figure).

The structural theme of repeated, cysteine-rich sequences reappears within the human EGF receptor. Its extracellular domain contains four cysteine-rich repeated sequences of about 40 amino acids in length together with two of about half that size. The EGF receptor repeat units are enclosed within a larger duplicated sequence which involves the entire extracellular domain of the EGF receptor and seems to have led to the generation of six homologous repeat units⁸. Beyond these repeat units, however, the EGF receptor does not display significant sequence homology with either the EGF precursor or the LDL receptor.

The fact that the EGF precursor shares structural features with two cell-surface

molecules and membrane receptors have been reported. For example, the receptor for transepithelial transport of two immunoglobulin molecules (IgA and IgM) displays structural homology with its ligands⁹. In this regard, it is interesting to note that the precursor for a polypeptide homologous to EGF, transforming growth factor (TGF)-alpha, also possesses a putative transmembrane sequence in a similar position relative to the mature growth factor sequence¹⁰. However, the TGF-alpha precursor is considerably smaller than the EGF precursor and lacks cysteine-rich repeats.

Together, these findings suggest that the EGF precursor may be a membrane protein in addition to being the precursor of EGF. That idea is strengthened by the detection by Rall *et al.* of the unprocessed EGF precursor but not mature EGF in regions of the mouse kidney. It will be important next to determine if the precursor is actually a membrane protein, and if so, whether it is localized on the cell surface or remains within the cell. Should this molecule be located on the cell surface, determination of its function will present



receptors, including a hydrophobic domain which could anchor the precursor in the membrane, suggests that it has evolved from an ancestral cell-surface protein. Perhaps exon-shuffling events generated this family of nutrient and growth-factor receptors, with intragenic duplication events producing the repeated structural domains that evolved to serve best the specific needs of each protein. While the significance of the cysteine-rich repeat units for the EGF precursor and EGF receptor can at present only be guessed, they have been postulated to contribute to a multiply-looped, rigid LDL-binding domain in the LDL receptor².

Structural homologies between secreted

a major new challenge. It is possible that by regulation of protein processing, the EGF precursor serves different functions in different tissues. □

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* A clerical error generated a frameshift at position 3707 in the mouse EGF precursor sequence reported in ref. 4.

Possible functions of the homoeobox

SIR — Recent exciting findings by several laboratories have centred on the possible molecular mode of function of the *Drosophila* homoeotic gene products which appear to act as regulatory elements within the nucleus to determine the development fates of specific groups of cells¹⁻⁵. Of particular interest has been the observation of the "homoeobox", an intriguing structural element which is common not only to several genes in the *Drosophila* homoeotic gene complexes *Antennapedia* and *Bithorax*, but to other genes in *Drosophila* and in diverse other species as well³⁻¹⁰.

Although the function(s) of other genes which contain the homoeobox sequence is, at present, not known, it has been a tempting speculation that the putative protein domains encoded by the conserved homoeobox sequences may serve a common regulatory role in some aspect of pattern formation or "segmentation"; for example, Carrasco *et al.* speculate that the "homeo domain may give cells universal yet simple instructions, such as to stop dividing, to divide faster, to be considered determined, or to stop searching for new pathways of development"⁷. However, the fact that this domain has been so highly conserved in evolution suggests that it may serve a very basic conserved cellular function. We wish to point out that this function may not necessarily be directly related to the overall role of the gene product of which the homoeobox protein domain is a part.

It is a mundane but nonetheless inescapable fact of cellular life that translation occurs in the cytoplasm and that proteins with nuclear function must be transported into and/or sequestered in the nucleus. Thus, one possibility for a very basic generalized role of the homoeo domain might be in determination of the destination within the cell (the nucleus) of the gene product of which it is a part. There are, of course, other possibilities; for instance this domain might represent a generalized binding region for nucleic acid or for acidic proteins. The idea that the homoeo domain might simply represent a nuclear tag is generated by the recent work of Kalderon *et al.*¹¹, part of whose data so nicely decorated the cover of the 6-11 September issue of *Nature*. They showed that a region containing a run of five basic amino acids is solely responsible for nuclear localization of the SV40 large-T antigen; indeed, they found that replacement of the first Lys residue with a noncharged amino acid is sufficient to cause this protein to remain entirely cytoplasmic. One cannot help but compare this striking result with the fact that the key conserved feature of the 60 amino acid homoeobox protein sequences are three domains in which similar short runs of basic amino acids occur.

Consistent with the hypothesis that the homoeobox sequence serves as a design-

nator on nuclear destination is its location in the 3' exon of the *Drosophila* homoeo genes, which would give it an omni-present position in the variable gene products derived from the complex 5' splicing patterns of many of these genes. This hypothesis also offers a simpler explanation for the presence of multiple copies of similar sequences in the genomes of many species and obviates the necessity of invoking homologies in segmentation processes between organisms from diverse phyla. McGinnis *et al.*¹⁰, for example, have suggested that

If the conserved homoeo domain in fruit flies, frogs, mice, and humans is involved in segmental development, then it is possible that the segmentally organized animals in both the protostome and deuterostome classes had a common ancestor, and that the metameric body plan has evolved only once in evolution.

The phylogenetic soundness of this argument is open to question. Annelids and arthropods are fundamentally metameric animals in which the body is organized as a linear series of segments. In primitive forms, these segments and their repeated internal and external structures are very similar. In the evolution of insects these metameric segments have become highly differentiated from each other. Homoeotic genes can be viewed as having become progressively more elaborate as regulators of segment differentiation in this evolutionary lineage¹². While chordates do possess some serially repeated structures, most prominent of which are the somites, they are in no real sense metameric, as can clearly be seen from the most primitive group of chordates, the ascidians. While we agree that the protostomes and deuterostomes must share at some point a common ancestor, chordates are, in fact, not segmented animals and are fundamentally distinct from arthropods and other metameric animals both in body plan and in the developmental processes by which the body plan is achieved. The evolution of these developmental programs offers little evidence of meaningful homology¹².

We bring up this hypothesis for the sake of discussion; the answer of course will lie in data to come. Certainly a sequence of four to five basic amino acids is not a "universal" nuclear tag. Nuclear designator sequences for a number of other proteins have also been characterized; these comprise several different amino acid sequences placed in different places within the parent proteins¹³⁻¹⁵. In any event, if the homoeobox sequence indeed functions as a designator of nuclear destination, the possibility is testable by experiments such as those of Hall *et al.*¹⁵, who showed, by constructing fused gene products, that sequences from the nuclear protein yeast mating type factor Mat $\alpha 2$ are also able to confer nuclear localization on heterologous proteins. Finally, let us note the

reported homology between the homoeo domain protein sequence and both of the yeast mating type factors, Mat $\alpha 1$ and Mat $\alpha 2$ (refs 8, 9); certainly there is little argument for *Saccharomyces* as a segmented organism.

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SIR — The current research into DNA sequences involved in segment pattern formation in invertebrates¹⁻⁴ and their putative homologues in vertebrates⁵ raises a variety of questions concerning, first, the nature of the development process in vertebrates and invertebrates and, second, the nomenclature used to describe this process and its associated genomic sequences. Comparisons between invertebrate and vertebrate development may be, at this early stage, invidious, and we would like to take this opportunity to state the reason why we feel it to be dangerous to develop an all-encompassing paradigm for the problems of segmentation in higher organisms.

Much interest is concentrated on the homoeobox sequence common to several homoeotic genes in *Drosophila melanogaster*⁶. Similar sequences are found in vertebrates^{5,7,13}. The homoeobox sequence is also found in a *Drosophila* segmentation gene (*fushi tarazu*) which does not confer a homoeotic mutant phenotype^{6,8}. While the exact role of this sequence is unclear, in both invertebrates and vertebrates, it clearly shows a common association with the process of segmentation or metamorphism in arthropods.

Animals which are truly metameric or show metameric stages are confined to the invertebrates. Vertebrates show limited segmentation which is confined primarily to the muscles of the trunk and associated skeletal and nervous systems. Indeed, it is possible that vertebrate segmentation has developed independently of vertebrate groups in evolution^{9,10}. This raises the question of whether the homoeobox sequences, found in both invertebrates and vertebrates, plays, as has been suggested¹¹,

a common role in a process whose only feature may be an axial arrangement of tissues. It would thus also be premature to surmise that, if such homoeobox sequences are truly ancestral, the conservation of such a sequence reflects a common ancestral lineage for invertebrates and vertebrates in the development of segmentation controlled by a common, homologous DNA sequence.

However, alternative possibilities exist. Leaving aside convergent evolution, an ancestral homoeobox sequence may have been usurped or recruited for different functions in different morphogenetic processes in separate evolutionary lineages, and although invertebrates and vertebrates possess a common homoeodomain, it may possibly be involved with quite different aspects of development. Given a possible DNA-binding role for the homoeobox sequence¹² the homoeo-product may have a common function within different morphogenetic processes in separate evolutionary lineages, thus explaining its continued conservation.

Therefore, it seems to us somewhat premature to associate homoeobox sequences wholeheartedly with vertebrate segmentation and the control thereof. There is clearly an inherent danger in establishing such a model. It is too soon to rule out the role of homoeoboxes in morphogenetic processes other than segmentation.

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T-cell receptor genes and ataxia telangiectasia

SIR — The nature of the T-cell antigen receptor has been recently discussed in *News and Views*¹. The genes for the β chain of the T-cell receptor have been cloned and mapped and human chromosome 7, bands 7p13-21², while the α -chain genes still await chromosomal assignment. We wish to draw attention to some observations which might indirectly bear on the latter issue as well as on the pathogenesis of immunodeficiency in human disease.

It is known that in lymphocytes from individuals with the chromosomal instability syndrome ataxia telangiectasia (AT), 7-14 translocations appear to be a nonrandom event (their frequency is more than 40 times higher than expected)³⁻⁶. Break-

points on these chromosomes are also highly nonrandom, involving bands 7p14 (consistently with the position of the T-cell receptor β -chain genes), 7q35, 14q12 and 14q32 (consistently with the known position of the immunoglobulin heavy-chain genes). The relative frequencies of breaks at 7p14 versus 7q35, and at 14q12 versus 14q32 are 1:1 and 3:1, respectively³⁻⁶. It is reasonable to speculate that the spontaneous breakages at bands 7p14 and 14q32 in AT lymphocytes originate from faulty rearrangements of the T- and B-cell receptor genes which map there. This also suggests that these genes are normally rejoined, during rearrangement, by the same repair mechanism which is defective in AT, thus providing a basis for the T- and B-cell immunodeficiency characteristic of this syndrome⁷. The fact that 7-14 translocations are found in T-lymphocytes might indicate that both chromosomes contain genes which rearrange in these cells. The nonrandom breakpoints at bands 7q35 and 14q12 may reflect the positions of two different sets of rearranging T-cell receptor genes, for example the α -chain and the so-called non- α genes¹.

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Medical versus animal antibiotics in resistance

SIR — In his letter on "animal antibiotics", A. H. Linton¹ says that "the present debate [on resistance] . . . hinges on whether or not subinhibitory, rather than prophylactic or therapeutic levels of antibiotics play the major role". I believe that subtherapeutic, rather than subinhibitory, levels fed to animals are central to the present debate. Even though there is laboratory work on subinhibitory levels, these low levels are not added to animal feeds.

As cited by Linton¹, there is a large reservoir of R plasmid-carrying indigenous flora in the intestines of animals², but these flora are not necessarily a significant source of resistance for human pathogens. For example, ampicillin-resistant *Neisseria gonorrhoeae* probably derives its resistance from human origins³.

The suggestion that the energy cost to bacteria of carrying R plasmids places them at a disadvantage has been negated in some studies, but has found support in others, as discussed by Timoney and Linton⁴. Rough cultures of *Salmonella choleraesuis* var.

kunzedorf, with associated reduced virulence, were found to be better recipients of R factors than were their smooth virulent parental counterparts.⁵

Richmond and (K.B.) Linton⁶ found that sewage from hospitals contained more resistant organisms than did domestic sewage. They concluded that the main selective pressure for tetracycline-resistant organisms was from medical rather than veterinary use of antibiotics.

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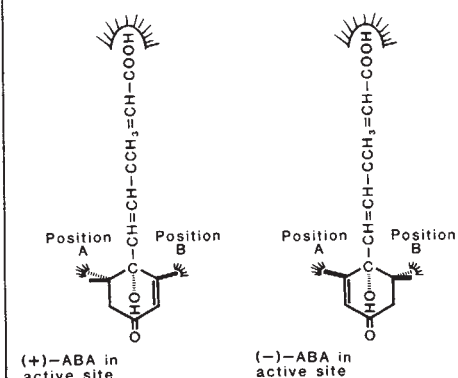
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Globulin flexibility worked out by hand

SIR — Pirie's recent note¹, about the flexibility of globulin molecules and the comfort of transposing left and right shoes, misses the main point of the original model². It proposes that (-)abscisic acid can fit into the normal active site of a receptor (affecting growth inhibition) which is normally filled by natural (+)ABA, provided that the 2'-methyl of the (-) can occupy the site of a 6'-methyl group of the (+), and a 6'-methyl of the (-) fits into the place which is occupied by the 2'-methyl of the (+). This transposition could occur without distortion.

It is analogous to putting the little finger of a left hand into the thumb of a right glove and one's thumb into the little finger's position. The procedure can be carried out without discomfort and provides an appropriate mode for the similar action of the two enantiomers of ABA.

Pirie puts his foot in; a more even-handed approach is suggested:



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Hotspot–migrating ridge interaction in the South Atlantic

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Short-wavelength geochemical anomalies in the basaltic layer 2A occur along the South Atlantic mid-ocean ridge opposite Ascension, St Helena and Tristan hotspots, further evidence that preferential subcrustal flow channels develop between mantle upwelling zones (hotspots) and migrating ridge axes. Geochemical variability suggests that the plume along these channels would be schlieren-like rather than continuous.

MANTLE heterogeneities have been researched extensively since significant differences in radiogenic Pb and Sr isotope contents of lavas from Ascension and Gough Islands were discovered¹ and attributed to long-lived heterogeneities in parent/daughter U/Pb and Rb/Sr ratios in the source of these lavas in the mantle (~1.5 Gyr). The majority of basalts erupted along mid-ocean ridges are thought to be derived from a relatively uniform source depleted in the large-ion lithophile elements (LILE). On the other hand, the sources of magmas erupting at oceanic islands and shallow submerged volcanic platforms are heterogeneous, tending to be less depleted or even enriched relative to bulk earth LILE contents. Radiogenic Sr, Nd, Pb, He and Ar isotope systematics^{2–6} suggest that the trace element composition of mantle domains, associated with hotspots, range from values approaching that of the primordial Solar System to enrichments or depletions indicative of extraction and recycling of crustal materials on various time scales.

Two main classes of models have been derived to explain these observations. First, a random distribution of LILE-rich mantle domains of various sizes are embedded passively in a LILE-depleted convecting mantle matrix^{7–11}, although whether convection is taking place over the entire mantle or is limited to the upper mantle above the 670-km discontinuity is unknown^{11–14}. Sampling of these different domains by lavas produced by partial melting at different rates, equilibrium–disequilibrium conditions and mantle volumes account for the heterogeneities observed along the mid-ocean ridges and islands. This model is often referred to as the raisin or ‘plum-pudding’ mantle model.

Second, LILE-rich mantle plumes or blobs, dynamically buoyant, rise beneath hotspots (oceanic islands or shallow submerged volcanic platforms) and interact and mix with the LILE-depleted asthenosphere while penetrating it from below^{15–18}. Here, the blobs are considered as relatively long-lived and spatially fixed sources of LILE-rich material, whereas mid-ocean ridge spreading axes are considered as sinks of upper mantle materials^{19,20}. These upwelling zones seem to be fixed with <5 mm yr⁻¹ of relative motion worldwide, or <3 mm yr⁻¹ in better constrained areas such as the Atlantic²¹. These upwelling zones seem to be essentially continuous on time scales of 100 Myr, judging from resulting constructional volcanism^{21,22}. However, where geochemical monitoring of LILE-rich and depleted volcanic products has been measured in sufficient detail, such as the Faroes–Iceland²³ and the Walvis aseismic ridges²⁴, the upwelling of LILE-rich material seems to be cyclic on a shorter time scale of ~10–20 Myr, suggesting the rise of a chain of blobs rather than continuous plumes. Whether these blobs are representative of the deeper mantle or some intermediate zones or domains is also unknown²⁵.

Three types of hotspots are recognized within the framework of plate tectonics: intraplate-plate (for example, Hawaii¹⁵), ridge-centred (for example, Iceland¹⁶) and near-ridge (a hotspot

once on the ridge but the ridge has migrated away from it; for example, Galapagos^{19,20}).

It is well established that ridge-centred plumes produce large-scale isotope and trace-element gradients along the ridge, unusually intense constructional volcanism, elevation anomalies or time transgressive V-shaped ridges, apparently as a result of preferential flow of LILE-rich plume-derived material below the ridge axis acting as a sink^{16,26}. We explain the geochemical gradients by mixing and progressive dilution of the LILE-rich subaxial flow with the LILE-depleted asthenosphere, which normally would feed the ridge completely¹⁶. In contrast, if the ridge axis drifts away from the plume as in the case of the Galapagos Spreading Centre, the plume apparently tends to develop a preferential non-radial flow towards the migrating ridge-axis which continues to act as a sink^{19,20,27}. Lava compositions are still gradational along the ridge axis, symmetrical about the hotspot and the envisaged connecting channel, but the gradients along the ridge axis are not as long and the elevation anomaly is more subdued. An important question in this working model is how long the plume-source ridge-sink mixing interaction and channelling of the plume can be maintained as the ridge axis is migrating progressively further away, and to what extent fracture zones may affect or enhance such flow.

Geochemical observations

To study this question, we report here on trace-element variations along the Mid-Atlantic Ridge (MAR) in the South Atlantic, where hotspots are at least 400 km away from the ridge axis (Fig. 1). Plate reconstruction in the framework of relatively fixed hotspots suggests a westward migration of the ridge for at least the last 25 Myr^{21,22,28–30}.

Based on existing geomorphological and geophysical evidence and the location of near-ridge hotspots, three dredging expeditions between 1° and 47° S in the South Atlantic sought to determine the presence or absence of hotspot-related geochemical anomalies along the MAR axis (Fig. 1).

We first examined the area around the Tristan da Cunha–Gough Islands (30°–47° S, Atlantis II, 107) where the shallowness of the ridge^{31,32}, the closeness of these two islands to the ridge (~450 km) and the presence of two parallel linear zones of earthquake epicentre activity just south of the 35° S fracture zone³³ (suggesting overlapping rifts or a recent rift jump, a configuration similar to Iceland) predicted that a geochemical anomaly might be found there.

The possibility of finding a geochemical anomaly between 7° and 10° S near Ascension Island was then investigated (10°–20° S, Endeavor 061). Previous observations^{34,35} indicated: (1) a change of strike of the MAR axis between 8°–9° S (ref. 35), an anomalous elevation³⁴ and instead of a rift the presence of an elevated crestal block (similar to the Reykjanes Ridge³⁶, or the Galapagos Spreading Centre²⁷); (2) a gap in earthquake epicentre activity³³, suggesting a more ductile crust (as in the

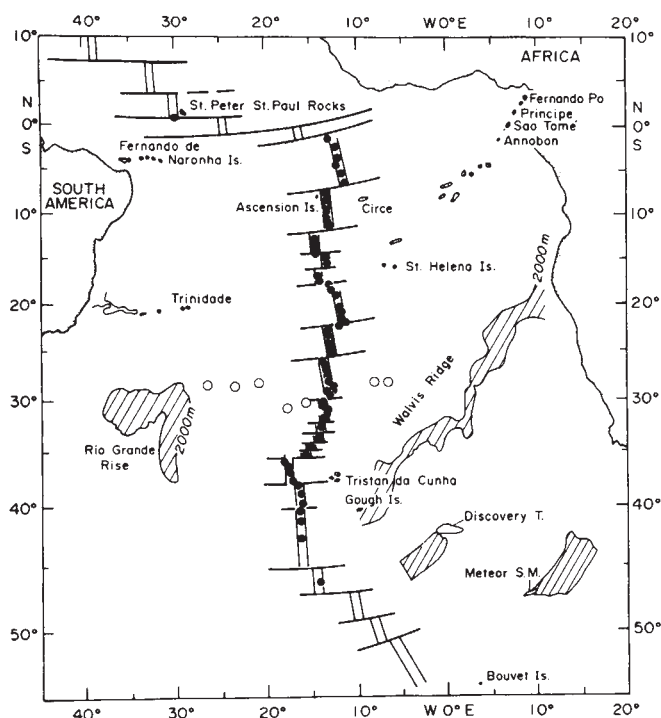


Fig. 1 Location of dredged samples along the MAR used in this study (closed circles). Location of DSDP Leg 3 drill holes are shown in open circles.

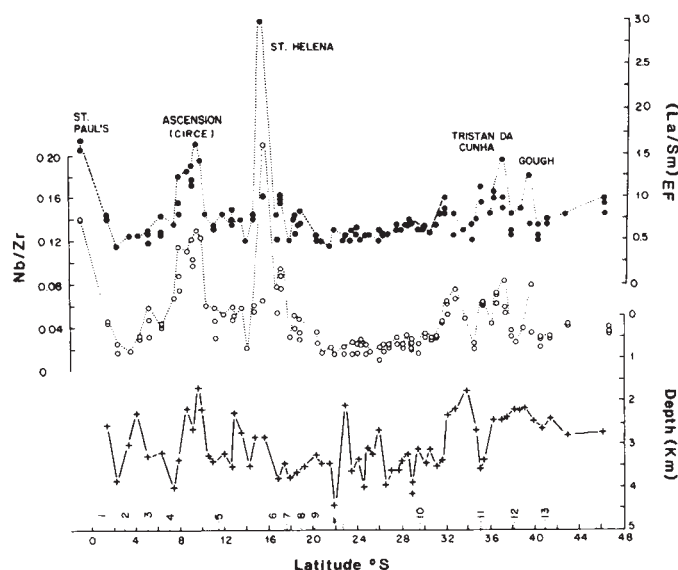


Fig. 2 (La/Sm) normalized to chondrites (closed circles), Nb/Zr ratios (open circles) and depth of sample recovery (crosses) as a function of latitude along the MAR Axis. Sample locations shown in Fig. 1. Note the good correspondence between Nb/Zr and La/Sm and large variability within anomalous spike regions (La/Sm > 0.7) relative to normal ridge segments (La/Sm < 0.7). Note the short wavelength elevation anomaly over Ascension (Circe) spike and the broader elevation anomaly high over the Tristan-Gough region. Position of fractures are shown as dotted lines with numbering system corresponding to: 1, Chain; 2, Charcot; 3, Fernando; 4, Ascension; 5, Gabon; 6, St Helena; 7, Bonaparte (named during Cruise ENO61); 8, Hotspur; 9, Martin Vaz; 10, Rio Grande; 11, 35° S; 12, Tristan; 13, Gough. The fracture zones near 23° S, 26° S and 46° S are unnamed. The exact sample location, depth of recovery and the geochemical data shown in this figure can be obtained by writing to J.-G.S.

northern part of the Reykjanes Ridge³⁷, where there is a geochemical gradient¹⁶⁻¹⁸); (3) a possible net deviation in the square root of time-versus-subsidence depth curve at this latitude^{34,38} and the presence of a large truncated topographic bulge 450 km east of the MAR over ~20–25 Myr-old sea floor (the Circe Hotspot, named after the Circe Expedition of Van Andel and Heath³⁴); (4) the presence of a transverse east-west ridge on the lower eastern flank of the MAR on 40-Myr sea floor and offset of magnetic anomaly lineaments³⁴, suggesting rift jumps, commonly associated with hotspots³⁹. We interpret these features as characteristic of a ridge near a hotspot, here, possibly the Circe hotspot rather than Ascension Island. We make no prediction for the MAR segment facing the 800-km distant St. Helena hotspot, as no ridge elevation anomaly or unusual geophysical feature was apparent in the region.

The third cruise (20°–30° S, Endeavor 063) was in a part of the MAR predicted to be 'normal' (from DSDP Leg 3 sampling across the ridge^{40,41} and lack of an anomalous ridge elevation^{31,32}).

Basalts from dredge-haul intervals of 40–100 km are generally fresh, often glass-rimmed and are believed to be of zero or near zero age, though the control is not as good as the North Atlantic as the location of the ridge axis is poorly known and the weather was particularly bad during the sampling over the Tristan Platform region. All the basalts can be classified as quartz or olivine normative tholeiites, with the exception of one sample (15.46° S), a nepheline normative basalt. Petrological and geochemical studies have been completed on these samples and will be reported separately for the three cruises (ref. 24; H. Sigurdsson and co-workers and J.G.S. and co-workers, in preparation). In this paper we focus on the variation of La/Sm and Nb/Zr ratios. These two LILE-ratios are closely related and good indicators of mantle source derivations, evident from the similarity with the ⁸⁷Sr/⁸⁶Sr in the North Atlantic Profile^{42,43}. Anomalously high (La/Sm)_N (> 1) and (Nb/Zr) ratios (> 0.06) are characteristic of LILE-rich mantle domains. Figure 2 shows that, in contrast to the large-scale gradient observed in the North Atlantic over the ridge-centred Iceland and Azores hotspots, only spike-highs in the LILE-ratios are observed at the approximate

latitude or mantle flow line of Circe, St Helena, and Tristan da Cunha-Gough near-ridge hotspots. These South Atlantic anomalies are characterized also by large local compositional variability as expressed in single dredge hauls (Fig. 2). Relative variability within the MAR segments comprising the three geochemical anomalies are also greater than the 20°–30° S normal ridge segment. The (La/Sm)_N means and % standard deviations of the 0°–12° S, 12°–20° S and 30°–40° S ridge segments are 0.81 ± 43 , 0.86 ± 58 and 0.83 ± 27 , respectively, whereas the 20°–30° S normal ridge segment averages 0.59 ± 13 .

The Circe spike anomaly centred at 8°–10° S is well delineated as the sampling interval chosen was small, significantly smaller than the half-width of the anomaly. This geochemical anomaly seems to be confined to a linear ridge segment bounded by the Ascension and Gabon Fracture Zones. It is accompanied also by a small anomaly in the ridge elevation, reflecting the progressive disappearance of a rift and the development of a blocky type of ridge crest resulting from constructional volcanism peaking at the same place as the geochemical anomaly. The case of the St Helena spike anomaly at 15°–16° S (just north of St Helena F.Z.), however, has too coarse a sampling density to define the half-width of the anomaly with any reliability at this time. There is no anomalous ridge elevation and a rift is present.

In the Tristan-Gough platform region, the anomaly is broadly distributed and significantly larger than the sampling intervals, except on the southern part. Several small spikes may be present throughout this region. The wavelength between these spike maxima is irregular and differs from the spacing between Tristan and Discovery hotspot trails trending northeast and ending approximately at the 25-Myr isochron²⁸. Thus, they are not located exactly opposite the hotspots if projected along the spreading flow line inferred from plate reconstructions. The spikes on the MAR axis are displaced southward by ~1° with respect to the position of the hotspots projected on the MAR

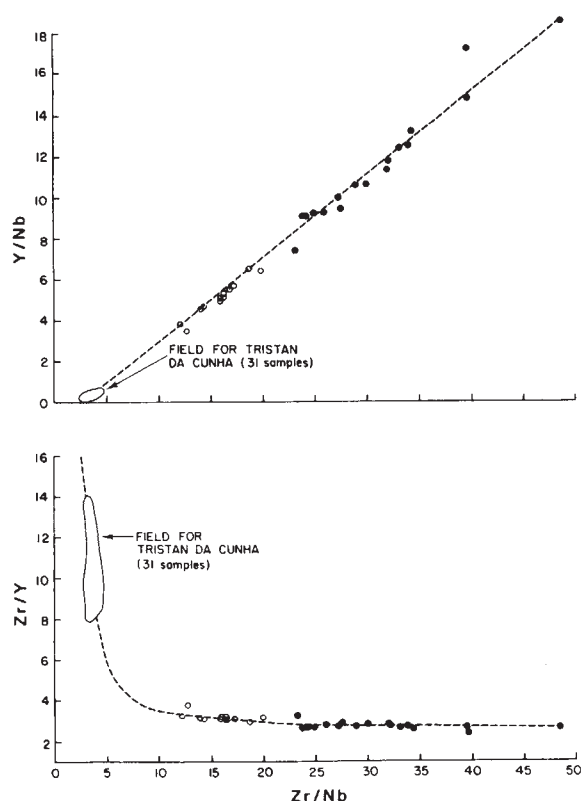


Fig. 3 Variations in Y/Nb and Zr/Y with Zr/Nb ratios in MAR basalts between 32° S and 46° S. Open circles are enriched samples within the spike-highs and closed circles normal ridge basalts. Dashed curve (or line) is calculated from a binary mixing model between a Tristan da Cunha plume source and a depleted asthenosphere source (see ref. 24 for details and source of data).

axis along the spreading flow lines; the 31.5° N spike has no hotspot counterpart on older sea floor. It is interesting to note that the ridge is migrating southwest at a rate of 2 cm yr⁻¹ relative to the hotspots²⁹. However, our preliminary sampling interval along the MAR is too large relative to the apparent wavelength of the spikes to decide whether these spikes are real or are random variations within the broad anomaly-high over the Tristan platform. Finally, the position of the geochemical spikes in the Tristan region do not show any systematic relationships to the location of fracture zones.

The ridge segment between 20° and 30° S is characterized by essentially LILE-depleted basalts and a rifted crest morphology. The depth of the MAR axis is also greater and shows less variation in this region of the South Atlantic (Fig. 2). The (La/Sm)_N and Nb/Zr ratios tend to increase slightly toward the south, irregularly and without relation to fracture zones.

Hotspot-ridge interaction

Our geochemical results suggest that the mantle anomalies associated with the off-ridge South Atlantic hotspots are sufficiently large, or mantle flow patterns are such that they can influence the source of eruptive products along MAR segments in their closest vicinity, here as distant as 450–800 km. It is significant also that our three first-order geochemical predictions, based on morphological and geophysical observations, have been confirmed; that is, a geochemical anomaly was found on the ridge segment nearest to Circe and the Tristan-Gough region, whereas the 20°–30° S segment was essentially normal. Thus our first-order test is conclusive. We also noted that there were no morphological indications suggesting an anomaly at the approximate latitude of the St Helena hotspot. However, on the basis of the model of migrating ridge sink-hotspot sources and our previous near-ridge hotspot case study of the Galapagos,

there was an indication that unusual ridge elevations may become more subdued, perhaps to the point of disappearing, with geochemical gradients becoming shorter as ridges migrate away from hotspots. Thus, the presence of the St Helena anomaly suggests that constructional volcanism is likely to disappear first, whereas some anomalous plume material may still reach the migrating ridge.

Comparison with the North Atlantic also suggests that if mixing of LILE-rich plume and LILE-depleted asthenosphere material causes the observed geochemical anomalies, then this process becomes more irregular and also less efficient as the ridge migrates away from the hotspot, evident from the lack of regular gradients and greater local variability over the South Atlantic geochemical anomalies. Either mantle heterogeneities become smaller and more irregular in shape or the mechanism responsible for the gradients about ridge-centred hotspots is no longer operational. The suggestion in the random 'plum-pudding' mantle model that increasing spreading rate may sample larger volumes of such mantle by partial melting¹⁰ and/or enhance the development of magma chambers where homogenization can take place before eruption^{9,44} does not hold in the South Atlantic, because the spreading rate increases southward from the North to the South Atlantic from 2–4 cm yr⁻¹ full rate. We suggest instead that local variability is more related to the presence or absence of a hotspot and its distance from the ridge, evident in the South Atlantic from the local uniformity along the 20°–30° S segment where there is no hotspot and the large variability in the spike-highs along segments opposite the Circe, St Helena and Tristan-Gough hotspots. This is evident also in the North Atlantic along the regular gradients near the ridge-centred Iceland and Azores hotspots, where local variability per unit length of the ridge is usually smaller.

We next need to test the proposed binary mixing and connection between the short wavelength anomalies on the ridge and nearby off-ridge hotspots. This can be done both geochemically with isotope ratios and with incompatible trace-element ratios. If the variations in the LILE ratios within the spike-highs have not been affected significantly by partial melting and magma emplacement processes and reflect binary mixing between ridge and hotspot source, each spike should also produce a distinct mixing array in trace element co-variation diagrams, pointing toward the particular signature of the corresponding near-ridge hotspot with which it is connected. The more distinct the geochemical signatures of these hotspots, the more reliable is the test. Available data from our study of the Tristan da Cunha system indicate that indeed a connection may exist²⁴. Both Tristan Island basalts and corresponding samples from the MAR in the region define a good mixing curve (Fig. 3) and thus seem to pass the test, despite the likelihood that these ratios may have been affected partly by smaller degrees of partial melting, particularly for the Tristan alkali lavas used in this test²⁴. An important question is the physical form of the flow and mixing toward the MAR, which may occur in the form of solid, solid-liquid, or melts only. The lack of greater dispersion about the mixing trends in Fig. 3 tends to eliminate melts (see ref. 20).

Mantle flow patterns

Hotspots are considered here as an upwelling source of LILE-rich material and ridges as sinks which, in the absence of hotspots, are fed passively with LILE-depleted asthenospheric material as plate divergence takes place. It is of interest to evaluate how the new South Atlantic data can provide constraints on probable upper mantle flow patterns beneath the region.

We believe that the modelling and surrounding arguments must remain simple, both because resolution is limited by the sampling density of this first-order survey which is coarse and essentially one-dimensional whereas the flows are not, and because of problems inherent in the sampling method of using melts as probes of upper mantle heterogeneities. These problems include the relative length scale and geometry of the melting

zone compared with that of the mantle heterogeneities sought, operative degrees of melting and modes of melt segregation and pathways taken, whether magma chambers are present or not (as original heterogeneities can be obliterated by convective mixing in magma chambers⁴⁴) and the individual length scale of such magma chambers relative to that of the sampling intervals.

Ignoring these problems, Fig. 4 illustrates schematically sea-floor spreading/asthenosphere flow models often considered in the literature. Model 1 considers the ridge to be over the limb of two diverging convective cells (active symmetric spreading). The point source of LILE-rich material associated with the hotspot would be deflected away from the ridge axis. In symmetric spreading model 2, the flow to the ridge axis is essentially subvertical and takes place passively in response to plate divergence. In both cases, the near-ridge hotspot activity would remain undetected at the ridge axis. Accordingly, we reject these two spreading-flow models for the South Atlantic.

On the other hand, models 3–5 could generate LILE-rich spikes at the ridge axis. Model 3 considers a passive subhorizontal return flow probably representing the case of a relatively thin asthenosphere. Material from the near-ridge upwelling zone would be deflected toward the ridge axis, smeared and dispersed as it penetrates the asthenosphere, a zone subject to high shear deformation⁴⁵. The plume need not have been centred previously on the ridge axis in this model, but an associated elevation anomaly might not be explained as readily.

Model 4 is the near-ridge upwelling zone describing best the kinematics of plates with respect to fixed hotspots in the South Atlantic and the southwest migration of the MAR^{21,22,28–30}. The LILE-rich upwelling zone (hotspot) was on the ridge at time t_0 (25–80 Myr according to fixed-hotspot/migrating-ridge reconstructions); subsequently the ridge migrated away from the hotspot to its present position (t_1). A sublithospheric channel was established between the hotspot and the ridge axis acting as a sink; the sublithospheric channel extended progressively with time, thus maintaining a geochemical and elevation anomaly at the ridge axis at the latitude of the hotspot. The geochemical variability observed in the spikes at the ridge axis could be explained if the material from the plume were schlieren-like along the connecting channel, rather than continuous. Magma chambers beneath the ridge axis in these regions seem to be ephemeral or non-existent and mixing conditions are poor. Also the elevation anomaly at the ridge axis would subside as the ridge migrates away from the hotspot.

Model 5 illustrates the case of a random distribution of the LILE-rich mantle domains riding passively within the flowing asthenosphere (passive or active; see arrows in Fig. 3), that is, the 'plum-pudding' model. This model could produce the three geochemical anomalies observed and cannot be rejected readily. However, the probability of three such enriched domains taken from a pool randomly distributed over the globe and occurring on the ridge axis just opposite the three other off-ridge hotspots, and not elsewhere, must be very small.

Additional tests

We describe briefly two further tests to support the hotspot source/migrating-ridge sink and channelling connection model.

First, there are two other important hotspots which could have affected the MAR in the South Atlantic: Trinidad and Fernando de Noronha^{21,22} (Fig. 1). Both are located far from the ridge, Trinidad being about 2,300 km on the west, which, if projected along a spreading flow line, would fall around 21° S where no geochemical anomaly is apparent. This hotspot is therefore too distant or was never close enough to have sensed or affected the MAR. The track of this hotspot is likely to have been under South America for the last 120 Myr, had it been active all this time^{21,22}. The MAR ridge axis was never on this hotspot and could not have sensed it and be deflected towards it, in agreement with the geochemical evidence. Morgan²¹, however, predicted the track of Fernando de Noronha to run essentially parallel to the MAR between 5° N and 5° S and to

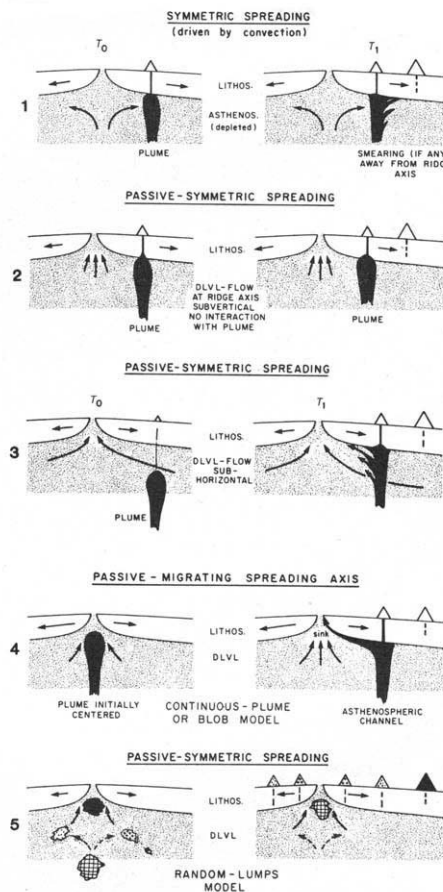


Fig. 4 Schematic representation of mantle flow-plate motion models which cannot (models 1 and 2) or can (models 3–5) explain the short wavelength La/Sm (or Nb/Zr) anomalies shown in Fig. 2. DLVL, depleted low velocity layer (depleted asthenosphere); lithos, lithosphere. Asthenospheric flow lines in model 5 are for passive spreading (full-line arrows) and active spreading (dashed arrows).

be close to or on the MAR 150–90 Myr, had it been active all this time. Projection of the present location of the Fernando de Noronha hotspot along the spreading flow line would place a possible anomaly just south of the Chain F.Z., where there is indeed an indication of a slight enhancement in the La/Sm at 1° S and near St Peter and St Paul Rocks (Fig. 2); thus it could be that these two anomalous stations might be related to the Fernando de Noronha hotspot track. Our sampling is too sparse for a proper definition of this possible anomaly; nevertheless, the above two observations and the similarity of Pb isotopes of Fernando de Noronha⁴⁸ and St Peter and St Paul Rocks⁴⁷ are consistent with predictions of the model.

Second, the connection between the near-ridge hotspot and the spikes along a sublithosphere channel can best be tested isotopically as the Ascension, St Helena and Tristan hotspots and the depleted asthenosphere have very distinct signature in three-dimensional Sr, Nd and Pb isotopic space². Any mixing between the LILE-depleted source and the particular hotspot, as suggested by our working hypothesis, should produce distinct binary mixing trajectories in such three-dimensional isotopic space. The mixing trajectories need not be in the mantle-plane², but could have a certain curvature depending on the Pb, Sr and Nd concentration of the end-member reservoirs, or scattered in a manner dependent on the internal uniformity of the end-member reservoirs²⁰ and on whether mixing occurs in a solid or melt form. The distinction between these hotspots is apparent also in isotope Pb 207/204 or 208/204 versus 206/204, as the Tristan-Gough hotspot has rather high 207/204 or 208/204, and deviates markedly from the general mantle array formed by

mid-ocean ridges and other oceanic islands (or ~ 1.6 yr secondary isochron)^{6,18,48}. We are investigating presently these tests, which have been proposed independently⁴⁹.

In conclusion, we believe that the working model of hotspot source-migrating ridge sink and channel connection shows sufficient systematics to be followed up by additional tests in other areas, or by further definition in the South and central Atlantic. We believe also that isolated anomalies in some areas, such as near the East Pacific Rise around 9°N ⁸, or on the SW Indian Ridge east of Bouvet Island⁵⁰, which have prompted respectively the random plum-pudding working hypothesis and

the variably-veined mantle model, deserve more scrutiny within the framework of the fixed hotspot/migrating ridge model, because it is possible that these isolated anomalies might have been produced by some more distant hotspots in these regions. These two types of models need not be mutually exclusive.

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Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells

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Co-cultivation of Agrobacterium tumefaciens with plant cells leads to the induction of circular copies of the T-DNA segment of the large tumour-inducing (Ti) plasmid in the bacterial cells. These circular molecules are presumably intermediates in DNA transfer from the A. tumefaciens genome to the plant cells. In support of this suggestion, the junction of the T-DNA circles occurs precisely in the 25-base pair terminal sequence involved in T-DNA transfer.

THE bacterium *Agrobacterium tumefaciens* transfers a particular DNA segment, the T-DNA, from its large Ti plasmid to plant cells. The expression of T-DNA after its integration into the plant genome results in crown gall, a tumorous growth^{1–3}. Recently, this system has been modified to allow the transfer of any DNA sequence without interfering with normal growth and differentiation of the transformed plant cells^{4–6}; it offers potential for the study and manipulation of plant gene expression^{7–12}. Here we investigate the DNA transfer process itself.

A 25-base pair (bp) sequence has a *cis*-acting function and directs T-DNA transfer from the nopaline Ti plasmid¹³; it is found as a direct repeat at the ends of the T-DNA region of the Ti plasmid and the borders of the T-DNA in the plant genome end at or very close to this sequence^{14,15}. Removal of the left copy of the sequence does not affect T-DNA transfer, but removal of the right copy abolishes tumour formation^{13,16–18}.

Reintroduction of the 25-bp sequence to a right border deletion promotes T-DNA transfer (assayed by tumour formation); furthermore, the reintroduced sequence was fully active only in the orientation found normally in the Ti plasmid¹³.

The mechanism of this DNA transfer is unknown. *Agrobacterium* itself is thought not to enter the plant cell; thus, the entire Ti plasmid or the T-DNA alone must be transported across the bacterial and plant cell membranes. We describe here the first isolation and characterization of T-DNA intermediates in the transfer between *Agrobacterium* and the plant cell.

Two independent methods were used to search for possible T-DNA intermediates. If a copy of the T-DNA is excised from the Ti plasmid during the transfer process, it might be detected by either λ *in vitro* packaging or plasmid rescue. The first method is sufficiently sensitive to detect possible intermediates if excess of both unaltered T-DNA in the original Ti plasmid and total

Agrobacterium and plant DNA are present. The method should also allow quantitation of intermediate structures relative to the T-DNA segment before any transfer events. Thus, we enlarged the T-DNA region of the nopaline Ti plasmid C58 to 47 kilobases (kb) in size by the insertion of the transposon Tn7 and the cosmid p3030 (Fig. 1); the resulting T-DNA region is almost the size of normal λ DNA and should therefore be packageable because of the *cos* site in the inserted p3030 portion. Intermediates of either tandem arrays of this T-DNA or circular T-DNAs would be substrates for the packaging reaction and packaged particles could then be used to transduce *Escherichia coli*, with those cells containing DNA with the cosmid and the Tn7 drug resistance markers being selected specifically. As λ packaging systems normally produce $\geq 2 \times 10^8$ plaque-forming units per μg λ DNA, the method should allow the recovery of packageable substrates in a large population of nonspecific DNA.

The second independent method, plasmid rescue, was designed to isolate circular T-DNA intermediates in particular. This strategy is based on the Ti plasmid pGV3850 (ref. 4), which has a short T-DNA region containing the left and right borders flanking the cloning vehicle pBR322. Thus, if T-DNA circular intermediates form by a joining between the T-DNA ends, they may be isolated after direct DNA transformation of *E. coli* by simply selecting for the ampicillin resistance marker of the pBR322 portion of the pGV3850 T-DNA. If such circles exist, they would be obtained specifically with total untreated *Agrobacterium* DNA, that is, without any cutting or ligation steps.

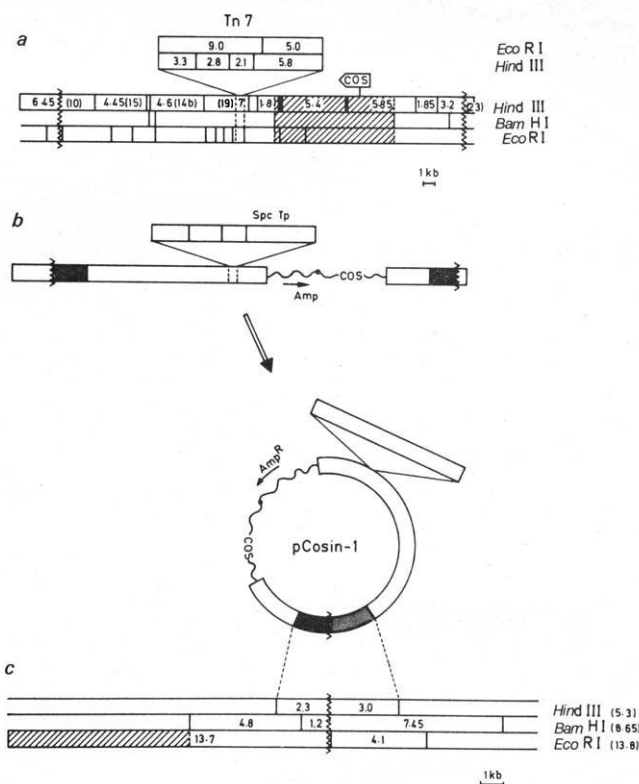
We sought to produce conditions where T-DNA intermediates might occur. Transcription of the virulence region of the Ti plasmid, essential for tumour induction, is induced specifically within a few hours of co-cultivation of *Agrobacterium* with regenerating plant cell protoplasts (ref. 19 and S. Stachel and E. Nester, in preparation). As co-cultivation is an efficient and reliable method for isolating plant cells transformed with *Agrobacterium*^{5,6,8,20}, it was used in the experiments reported here.

Cosmid rescue of T-DNA intermediates

Figure 1a shows the restriction map of the T-DNA region of the Ti plasmid pTiCos7. To produce tandem T-DNA arrays or T-DNA circular structures, there must be an earlier joining between the ends of the T-DNA. Following *in vitro* packaging of such substrates, a circular cosmid will be formed (Fig. 1b). Assuming that the T-DNA ends are joined very close to the 25-bp terminus sequence which defines the limits of the T-DNA, the size of the expected restriction fragments spanning such a junction can be predicted (Fig. 1c).

Agrobacteria containing pTiCos7 and plant cells were co-cultivated and DNA was prepared from either *Agrobacterium*-enriched or plant cell-enriched material; these DNAs were used as substrate for the λ *in vitro* packaging reaction (Table 1). Significant numbers of cosmids are obtained only after co-cultivation; furthermore, the number of cosmids detected increases with the length of time of co-cultivation (Table 1). Bacteria cultured in the same conditions without protoplasts never produced significant numbers of cosmid clones (data not shown).

To determine whether the packageable structures are formed in *agrobacteria* or plant cells, the amount of each type of DNA in each sample was estimated by the intensity of dot-blot hybridization to bacterial or plant DNA probes. The amount of bacterial DNA is approximately constant, whereas the plant DNA concentration varies. Higher numbers of cosmid clones are obtained with DNA from the bacterium-enriched fractions, and there are lower numbers of clones obtained with plant-enriched fraction DNA even when the bacterial DNA content is high. *Agrobacteria* closely associated with plant cells may contain modified DNA which is no longer an efficient substrate for *in vitro* packaging, or the T-DNA has been transferred already to the plant genome. The lower number of clones cannot be explained by an inhibition of *in vitro* packaging by plant DNA itself (see below).



and the rescued cosmid pCosin-1. **a**, Restriction map of the T-region of the Ti plasmid pTiCos7. Vertical lines, positions of the restriction enzyme sites; numbers, size (kb) of the different *Hind*III fragments; numbers in parentheses, *Hind*III fragments of the C58 nopaline Ti plasmid³⁹. The position of the *cos* sequence is indicated, as well as its polarity (arrow) of function for packaging^{23,24} in the T-region. Diagonal lines, location of the cosmid p3030 in this region; broken vertical lines, limits of the cosmid; jagged lines, T-region ends. The Tn7 (ref. 40) is located within a 0.7-kb *Eco*RI/*Sma*I fragment, delineated by broken vertical lines. The restriction map of Tn7 (ref. 41) is shown separately above; the numbers give the fragment sizes (kb). **b**, T-regions of pTiCos7 and one of the rescued cosmids pCosin-1. Wavy line, p3030; ●, pBR replication origin; Amp, ampicillin resistance; Spc, spectinomycin and streptomycin resistance; Tp, trimethoprim resistance; jagged lines, T-DNA border. The internal portions of *Hind*III fragments 10 or 23 ending just at the T-DNA borders are shown as stippled or black regions, respectively. **c**, Enlarged restriction map of the region of pCosin-1 formed by joining the T-DNA borders. All vertical lines, positions of the restriction enzyme sites; jagged lines, T-DNA borders; hatching, cosmid p3030; broken vertical lines, limits of p3030; numbers, size (kb) from the nearest restriction site up to the T-DNA border; numbers in parentheses, predicted sizes of the restriction fragments spanning the joint borders.

Methods: Cosmid p3030 (a derivative of the cosmid pHC79 (ref. 42) containing the *Eco*RI-D fragment of the yeast 2 μ plasmid inserted into its *Eco*RI site and a *Bam*HI fragment of the yeast *his3* gene in one of its *Bgl*III sites (B.H., unpublished data)) was inserted into the Ti plasmid pGV3132 by the reversed genetics method⁴³. First, the cosmid was inserted into one of the *Bam*HI sites of the *Eco*RI fragment 1 (ref. 39) of pGV1200 (E. Van Haute, unpublished data). The structure of the resultant intermediate-vector plasmid p3030-34 was verified by restriction analysis. The p3030-34 was mobilized⁴⁴ to *Agrobacterium* containing the mutant Ti plasmid pGV3132 (ref. 16), a derivative of the transfer-constitutive nopaline Ti plasmid C58 carrying the transposon Tn7 inserted in *Hind*III fragment 19. To select the carbenicillin-resistant and kanamycin-sensitive double recombinants exchanging the wild-type Ti sequence of pGV3132 by the modified Ti sequence of the p3030-34, two subsequent conjugations were performed to *Agrobacterium* strains cured of Ti plasmid, first to GV3101 (*Rif*^R) and then to GV3105 (*Ery*^R*Cm*^R) (ref. 16). The exconjugants are also resistant to spectinomycin, streptomycin and trimethoprim because of the Tn7. The T-region structure of the double recombinants was verified by restriction enzyme analysis and Southern blot hybridization.

Table 1 Induction of T-DNA intermediates as a function of time of co-cultivation

Time of co-cultivation (h)	Type of DNA in the mixture	Amount of respective DNA in reaction mix (ng)	No. of clones obtained by packaging	Normalized no. of clones per μg DNA
a, Bacterium-enriched fraction				
0	Total	1,010–1,020	1	
	Bacterial	1,000		
24	Total	1,050	662	630
	Bacterial	1,000		662
48	Total	750–800	2,970	3,712
	Bacterial	700		4,242
b, Plant-enriched fraction				
0	Total	1,200–1,300	1	
	Bacterial	1,000		
24	Total	2,000	62	31
	Bacterial	1,000		62
48	Total	1,300	559	430
	Bacterial	1,000		559

Protoplasts isolated from shoot cultures of *Nicotiana tabacum* SR1 (ref. 29) as described previously^{30,31} were washed³² and cultured in liquid K3 medium³³ at a density of 10^5 protoplasts ml^{-1} (50 ml in 14-cm-diameter Petri dishes). The cultures (at 28 °C) were grown in the dark for 2 days and thereafter in the light (500–1,000 lux). Three days later, cultures were infected and incubated further for different time periods. Bacteria were grown overnight in liquid TY medium (5 g l⁻¹ bactotryptone, 3 g l⁻¹ yeast extract) at 28 °C with shaking. Cells in early log phase (about $2 \times 10^8 \text{ ml}^{-1}$) were collected by centrifugation and concentrated 10 or 100 times, respectively, in TY medium. Protoplasts were infected with *Agrobacterium* for times (*t*) of 0, 24 and 48 h. To the samples *t* = 24 and *t* = 48, bacteria ($10 \times$ concentrated) were added at a multiplicity of 500 per protoplast, whereas 10 times more bacteria ($100 \times$ concentrated) were added to the *t* = 0 sample to compensate for bacterial growth during co-cultivation. At *t* = 0, collecting was immediately after mixing. Two different methods were used to prepare either bacterium-enriched (a) or plant-enriched (b) fractions. In the former, three volumes of 0.16 M CaCl_2 were added and plant cells pelleted by centrifugation for 10 min at 800 r.p.m. Bacteria from the supernatant were collected by filtration onto a Nalgene filter (pore size 0.45 μm). In the latter, an equal volume of 10 mM MgSO_4 was added and the mixture was centrifuged for 30 min at 18,000 r.p.m.. This pellet contains plant cells as well as attached bacteria, as the sucrose osmoticum of plant culture medium prevents the collection of free bacteria by centrifugation. Total DNA was prepared from both fractions as described by Dhaese *et al.*³⁴. From each sample, 5–20 μg total DNA was recovered and 1 μg DNA was used for *in vitro* packaging. The relative amounts of bacterial or plant DNA in each preparation were estimated subsequently by comparing the intensity of dot-blots hybridized with plant-specific probe (isolated fragments of dispersed *N. tabacum* repetitive DNA from the clone E10 (ref. 35) provided by M. Saul) or *Agrobacterium*-specific probe (total DNA of *agrobacteria* containing the pTiCos7) to dot-blots of a dilution series of known quantities of tobacco and *agrobacterium* DNA. The DNA was spotted onto nitrocellulose filters as described previously³⁶.

One of the rescued cosmids, pCosin-1, 47 kb in length and with one *cos* site, was used to assay the packaging efficiency in the presence of different concentrations of bacterial and plant DNAs (Table 2). Electron microscopy demonstrates that all the pCosin-1 DNA molecules (prepared from *recA*⁻ HB101 cells²¹) are monomeric (data not shown). The concentration of pCosin-1 DNA was chosen to produce a number of transductants close to that detected by packaging of the samples derived from co-cultivation experiments. Thus, 0.5 ng cosmid DNA was packaged in the presence of a constant amount of *agrobacterial* DNA (1 μg) and increasing amounts of plant DNA (0–1 μg) isolated from 5-day-old regenerating tobacco protoplasts. The concentration of plant DNA used might inhibit packaging only two- to fourfold, no more than the natural variation of the method (Table 2).

We obtain high efficiencies of packaging even with 47-kb monomeric circular substrates; pCosin-1 is packaged with 3–20% efficiency of that obtained with standard $\lambda\text{Cts857Sam7}$

Table 2 Determination of packaging efficiency in the presence of plant DNA

Amount of DNA (ng) added to the packaging reaction			No. of clones obtained	Packaging efficiency per μg
pCosin-1	<i>Agrobacterial</i>	Plant		
0.5	0	0	1,640	3.3×10^6
0.5	1,000	0	2,250	4.5×10^6
0.5	1,000	50	3,590	7.2×10^6
0.5	1,000	100	2,520	5.0×10^6
0.5	1,000	300	1,700	3.4×10^6
0.5	1,000	1,000	840	1.7×10^6
			Average efficiency	4.2×10^6

The preparation of λ *in vitro* packaging extracts, *in vitro* packaging reactions and transduction techniques are essentially those described by Arber *et al.*³⁷. About 1 μg of total DNA in maximum of 2 μl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was packaged in a reaction volume of 25 μl . After adsorption to the *recA*⁺ indicator bacteria W3110 (ref. 38) for 20 min at 37 °C, LB medium was added to dilute the packaging sample 10-fold. The mixture was incubated for an additional 30 min at 37 °C to allow expression of the ampicillin resistance and then plated using top agar onto LB plates containing ampicillin (50 $\mu\text{g ml}^{-1}$). For all experiments presented, the same packaging extracts were used so that the efficiencies are comparable. An efficiency of 1.5×10^8 plaque-forming units per μg was obtained with $\lambda\text{Cts857Sam7}$ DNA with the extracts used here.

DNA, agreeing with previous studies showing that circular λ monomers are as efficient as linear λ molecules using partially purified *in vitro* packaging extracts²². We suggest that the isolated T-DNA intermediate structures are circular monomers *in vivo*; however, we cannot exclude the possibility that a fraction of DNA is in multimeric circular or tandem T-DNA copies, which are also substrates for *in vitro* packaging.

We conclude that there is an increase in the number of packageable structures with time of co-cultivation, an indication for T-DNA intermediates which are probably formed in *agrobacteria* before transfer to the plant cell. They begin to accumulate before 24 h of co-cultivation, and at least some of them are circular monomeric T-DNA molecules.

Characterization of cosmid clones

All these clones grow in antibiotics selective for Tn7 (spectinomycin and trimethoprim) and cosmid p3030 (ampicillin), therefore these resistance genes are present. The restriction enzyme patterns of four clones were analysed both directly and after hybridization to probes specific for internal T-DNA sequences or sequences homologous to the T-DNA ends. These clones (pCosin-1, -2, -3, -4), derived from independent packaging reactions from two independent co-cultivation experiments, were identical (for example, see Fig. 2a,c). Restriction analyses showed also the presence of the 10.3-kb *Bam*HI fragment of cosmid p3030, the 2.8- and 2.1-kb internal *Hind*III fragments of Tn7, as well as fragments corresponding to the sizes expected for the hybrid fragments of both T-DNA and Tn7 or the cosmid (data not shown). Fragments corresponding to the expected sizes for the T-DNA borders *Hind*III-23 or *Hind*III-10, however, were not detected. Thus, the rescued cosmid clones are colinear with the T-DNA region of pTiCos7; only the T-DNA border fragments have changed. The restriction enzyme analysis indicates that the size of the cosmid clone is 47 kb, the exact size predicted for clones derived from joining at the T-DNA ends (Fig. 1b).

To investigate this joining, pCosin-1 was digested with *Hind*III and *Bam*HI and the separated fragments hybridized to *Hind*III fragments 23 or 10, which span the right and left borders of T-DNA. Instead of two Ti plasmid fragments corresponding to the left and right ends, we found only a single fragment in each digest which hybridizes with both probes (Fig. 2b); these results were confirmed by *Bgl*II, *Sal*I, *Kpn*I and *Pst*I digests (data not shown). We conclude that the T-DNA ends are joined; the sizes of the joint bands are precisely those

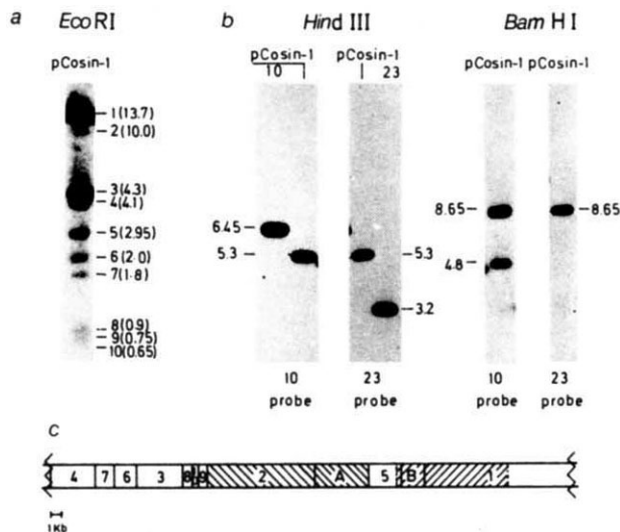


Fig. 2 Southern blot hybridization analysis of pCosin-1. **a**, Hybridization pattern of the cosmid pCosin-1 digested with the restriction endonuclease *Eco*RI and hybridized to a probe prepared from fragments covering the whole T-DNA region and pBR322. Numbers refer to the restriction map in **c**; numbers in parentheses give sizes of the different fragments (kb). **b**, Hybridization pattern of the cosmid pCosin-1 digested with either *Hind*III or *Bam*HI and hybridized to radioactive *Hind*III fragments 10 or 23 of the pTiC58. Clones pGV0369 and pGV0422 (containing *Hind*III fragments 10 and 23, respectively), are cloned in pBR322 (ref. 45) and digested with *Hind*III. Numbers refer to the size (kb) of the fragments. **c**, *Eco*RI restriction map of pCosin-1 with the Tn7 drawn in the T-region. Vertical lines, position of *Eco*RI restriction sites; left-hand hatching, Tn7; right-hand hatching, p3030; broken vertical lines, position of its insertion; jagged lines, T-DNA borders. Numbers refer to the numbers of fragments in **a**. A and B, fragments not visible in **a** as the probe used has too little homology to these fragments.

predicted if the T-DNA ends were joined close to the 25-bp repeat (Fig. 1c).

We performed further tests to determine whether the clones are representative of the numerous clones detected (Table 1). All of the 100 randomly-chosen cosmid clones were resistant to spectinomycin and trimethoprim, 85 of these colonies hybridizing to DNA from both the right or left T-DNA borders. Cosmid clones derived from control samples (zero time of co-cultivation or bacteria cultured without protoplasts) hybridize to only one of the two borders. These latter clones, as well as a proportion of those detected in the co-cultivation experiment, probably arise from packaging starting at the *cos* site internal to the T-DNA of pTiCos7. As λ packaging is polar^{23,24}, packaging starting from the *cos* site as oriented in the T-DNA region of Ti plasmid pTiCos7 (see Fig. 1a) would proceed leftwards to give packaged DNA carrying ampicillin resistance and the pBR replication origin of p3030, as well as Tn7 resistance markers. Other clones could arise also from the packaging of circular molecules carrying the above markers derived from low-frequency recombination events in the Ti plasmid; the efficiency of the λ -packaging reaction would allow the detection of such rare events.

Concentration of T-DNA intermediates

Assuming the Ti plasmid (200 kb) is 2.6% of *Agrobacterium* DNA (7.6×10^3 kb) and the T-DNA of pTiCos7 is 25% of the Ti plasmid, 1 μ g bacterial DNA is equivalent to 6.5 ng T-DNA. Assuming one packageable T-DNA copy per cell and a packaging efficiency of 4.2×10^6 cosmids μ g⁻¹ (Table 2), 6.5 ng should produce 2.7×10^4 cosmid clones. After 48 h, the bacterium-enriched fraction DNA produced 4,200 cosmid clones μ g⁻¹, corresponding to one packageable copy of T-DNA in 15% of the cells; for plant-enriched fraction DNA this estimate would be 2%. These calculations provide an estimate of the relative

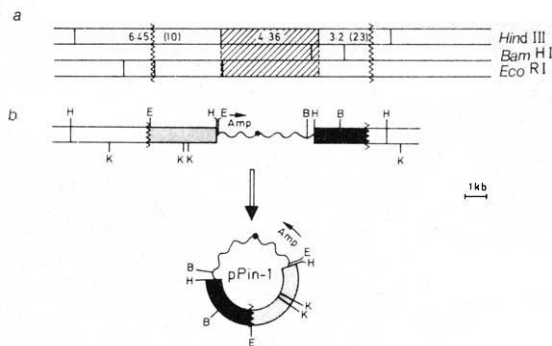


Fig. 3 Restriction map of the T-region of pGV3850 and the transformant pPin-1. **a**, Restriction map of the T-region of the Ti plasmid pGV3850 (non-oncogenic derivative of nopaline Ti plasmid C58) where the internal T-DNA genes are deleted and replaced with the cloning vehicle pBR322 (ref. 4). Vertical lines, restriction enzyme sites; numbers, size (kb) of the different *Hind*III fragments; numbers in parentheses, *Hind*III fragments of the C58 nopaline Ti plasmid³⁰; hatching, pBR322 in the T-region; broken vertical lines, its limits; jagged lines, T-DNA borders. **b**, T-region of pGV3850 and of one transformant, pPin-1. Wavy line, pBR322; ●, pBR replication origin; Amp, ampicillin resistance; jagged lines, T-DNA borders; vertical lines, positions of restriction enzyme sites. The internal portions of *Hind*III fragments 10 or 23 ending just at the T-DNA borders are visualized as stippled or black regions, respectively. Abbreviations: H, *Hind*III; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I. Plasmid pPin-1 was obtained following transformation of competent cells of *E. coli* strain MC1061 (ref. 46) with DNA prepared from *Agrobacterium* carrying pGV3850 co-cultivated in the presence of plant cells, with conditions described in ref. 47).

concentration of T-DNA intermediates based on the detection of circular monomeric T-DNAs.

Plasmid rescue of T-DNA intermediates

The T-DNA intermediate is either a linear tandem array, or a multimeric or monomeric circle, as all such structures would be packaged efficiently *in vitro* by λ extracts. We therefore assayed specifically for circular T-DNAs using the standard technique of DNA transformation. (Circular DNA is much more efficient than linear DNA when transforming *E. coli*²⁵.) As the transformation efficiency decreases drastically with increasing size of the transforming DNA²⁶, we used *agrobacteria* containing Ti plasmid pGV3850, which has a short T-DNA region with pBR322 sequences replacing the internal portion of the T-DNA (Fig. 3; ref. 4). Thus, we predict T-DNA circles to be 9.6 kb, allowing detection after transformation of competent *E. coli* cells. Such transformants would be ampicillin-resistant because of the pBR portion of the pGV3850 T-DNA.

Agrobacterium containing the Ti plasmid pGV3850 were co-cultivated with regenerating protoplasts for 48 h and total bacterial DNA prepared. Ampicillin-resistant transformants were detected following direct DNA transformation, without restriction endonuclease treatment or DNA ligation. These clones contain exactly the structure predicted for a joining between the ends of the T-DNA (Fig. 3). This demonstrates that some of the intermediates in the transfer process exist as T-DNA circles. Control experiments using DNA prepared from *agrobacteria* containing pGV3850 alone (without co-cultivation) never produced clones of circular T-DNAs.

To estimate directly the frequency of such circular T-DNA intermediates relative to the T-DNA in the original Ti plasmid population, the DNA prepared from co-cultivated *Agrobacterium* was used to compare the transformation efficiency of this DNA used directly (that is, without enzymatic cutting or ligation) with the efficiency of the same DNA digested with *Kpn*I (Fig. 3) and ligated before transformation. The latter reaction also produces circles of 9.6 kb from the original Ti plasmid, as a single *Kpn*I fragment within the T-DNA of pGV3850 carries the whole inserted pBR322 and some flanking sequences (Fig. 3). The number of clones obtained from the

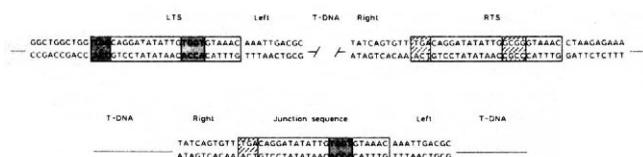


Fig. 4 Diagram of the 25-bp left terminus, right terminus and the junction sequence. The terminus and junction sequences are shown in the boxes. The nucleotide sequences from the adjacent regions of the nopaline Ti plasmid (C58 or T37) are given also. LTS, left terminus sequence; RTS, right terminus sequence; regions of variation between the left and the right terminus sequence are designated by stippled (LTS) or hatched (RTS) areas. In the junction sequence the regions homologous to the LTS or RTS are also stippled or hatched.

Methods: The *Hind*III fragments of three independent cosmid rescuants, pCosin-1, -2 and -3, spanning the joint borders were subcloned first in pUC8 (ref. 48). A small *Sac*II/*Eco*RI fragment (rendered blunt at the *Sac*II end) spanning the junction of the left and right T-DNA borders from all three clones was further subcloned into both the mp8 and mp9 M13 vectors⁴⁹, which were first cut by *Hinc*II and then by *Eco*RI. The nucleotide sequence was determined by the dideoxy-chain termination method⁵⁰. Both orientations of two subclones were sequenced, whereas in the third only one orientation was determined, as the sequence at the border junction could be read without ambiguity. The junction clone from the transformed pPin-1 derived from *Agrobacterium* carrying pGV3850 was sequenced by the method of Maxam and Gilbert⁵¹. pPin-1 was cut with *Eco*RI, kinased with radioactive ³²P then digested with *Bam*HI. The *Eco*RI/*Bam*HI fragment spanning the junction was isolated from an agarose gel and used directly for sequence analysis.

direct DNA transformation was 10% of those obtained after cutting and ligation. The former clones are T-DNA circles formed *in vivo* by specific interaction between the T-DNA ends and the latter are circles formed *in vitro*. As the size of the *in vivo*- and *in vitro*-formed circles is exactly the same, their efficiency of transformation should be comparable directly. Because the cutting and ligation reactions do not occur at 100% efficiency, this comparison gives only an upper estimate of the proportion of T-DNA circles relative to the intact Ti plasmid *in vivo*. Nevertheless, these data are in the same range of the estimates calculated from our λ -packaging experiments.

Junction sequence of circle intermediate

As the observed intermediates have arisen through a joining close to the T-DNA ends, we sought to determine both the nucleotide sequence at the junction site and whether the junction is the same in different isolates of intermediate structures. Four independent junctions were sequenced, three isolated from cosmid clones and one from the pBR plasmids; all were identical (Fig. 4). The junction occurs precisely in the 25-bp direct repeat normally found at the ends of the T-DNA region of the Ti plasmid. Furthermore, as there is nucleotide sequence variation between the left and right copies of the 25-bp sequence, the junction may be a hybrid of these copies. The 25-bp length of the sequence seems accurate as it is derived from comparison with four other terminus sequences from the octopine Ti plasmid^{27,28}. If it were only 22 bp starting after the TGA of the right copy (or TGG of the left copy), however, then the junction sequence would be derived entirely from the left copy. Mutation analysis of the sequences in and around the 25 bp will define its limits. Nevertheless, formation of the junction must have occurred by an interaction such as site-specific recombination in the 12 bp between bases 4 and 15 of the right and left copies of the 25-bp sequences.

Discussion

The sensitivity of λ cloning techniques used here allows the induction of T-DNA intermediates to be measured; the DNA transformation method not only confirms their presence but also suggests a probable structure, originating from circularization

of T-DNA during induction of its transfer. Such circles could produce tandem T-DNA arrays or multimeric circular structures suitable for the λ -packaging reaction. Monomeric (or dimeric) T-DNA circles derived from pGV3850 T-DNA containing pBR322 are the only possible DNAs which can be recovered by *E. coli* transformation to ampicillin resistance.

We believe that the T-DNA structures isolated here are intermediates in the transfer process because they occur in *agrobacteria* only during co-cultivation in the presence of plant cells under conditions where T-DNA transfer occurs; the concentration of these intermediates increases with time of co-cultivation. Also, the circular intermediate junction site occurs precisely within the 25-bp terminus sequence essential for T-DNA transfer. It will be interesting to determine whether the junction sequence *per se* is important for transport and/or integration of the T-DNA.

T-DNA circles may explain also the polar activity of the 25-bp sequence; reversing the direction of the right copy of this sequence reduces severely T-DNA transfer¹⁵. Thus, T-DNA circles could arise by site-specific recombination in the 25-bp terminus sequence at the right and left ends of the T-DNA; such recombination requires a correct orientation (polarity) of the 25-bp sequences.

Whether or not the observed structures represent the major T-DNA intermediate or even if they are only a by-product of the transfer reaction, they must be accounted for in any model of the mechanism of T-DNA transfer. Either the T-DNA circles themselves are transferred, or they serve as an intermediate in the generation of another molecule, for example by serving as a template to produce tandem T-DNA copies by rolling-circle replication in which T-DNA could be transferred as a linear molecule. Future experiments may distinguish between these alternatives.

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Regulation of a protein synthesis initiation factor by adenovirus virus-associated RNA_i

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A small RNA accumulating late in adenovirus infection is required for efficient protein synthesis, although not specifically for the translation of viral proteins. This RNA maintains the activity of an initiation factor catalysing the earliest step of polypeptide chain initiation.

SMALL RNAs have been implicated in translational control, but rarely has the RNA been well characterized and the phenomenon well authenticated. Progress has been hampered by the low abundance of these putative regulatory species in the cell and by their resistance to existing techniques of genetic analysis. Such obstacles do not exist, however, for the 'virus-associated' RNAs of adenovirus, VA-RNA_i and VA-RNA_{II} (refs 1-3). These RNAs, about 160 nucleotides long, are transcribed from closely spaced genes located near position 30 on the viral genome³⁻⁵. Both are detectable from early times of infection, but the rate of VA-RNA_i synthesis increases rapidly in the late phase so that this species becomes a dominant RNA in the cytoplasm of adenovirus-infected cells⁶. The VA-RNAs are capable of extensive intramolecular base pairing^{5,7,8}, and they also anneal with viral messenger RNA (ref. 9). A small proportion of the VA-RNA molecules in the cell occurs in ribonucleoprotein particles in combination with the La antigen^{10,11}, a cellular protein binding to short runs of 3'-terminal U residues^{12,13}. Like many La-reactive RNAs, the VA-RNAs are synthesized by RNA polymerase III (refs 6, 14, 15).

Involvement of these small RNAs in protein synthesis was first suggested by studies with mutant viruses such as adenovirus-5 (Ad-5) d1331. This virus fails to generate VA-RNA_i and proceeds normally through the early phase of the infectious cycle but is defective in production of late proteins¹⁶. The late viral mRNAs are present and normal in structure and can be translated *in vitro*, but are not translated efficiently *in vivo*. Another mutant virus, producing VA-RNA_i normally but failing to make VA-RNA_{II}, does not show the translational deficiency¹⁶. Similarly, in a transient expression assay, the synthesis of viral proteins was stimulated markedly when their genes were co-transfected into human cells with the VA-RNA_i gene, whereas the VA-RNA_{II} gene was much less effective¹⁷.

These data suggest a new function for RNA as a positive effector in translational control, but do not establish whether the translational action of VA-RNA_i is general or limited to viral mRNAs. To answer this question and to establish the mechanism of the translational effect, we developed a cell-free protein-synthesizing system from HeLa cells infected with the d1331 mutant.

Protein synthesis defect *in vitro*

Suspension cultures of HeLa cells were infected with either the mutant virus d1331 (producing no VA-RNA_i) or wild-type adenovirus-2 (Ad-2) virus, or were mock-infected; extracts were prepared in the late phase, 24 h later. Proteins were synthesized by the d1331 extract at only 1-5% of the level obtained with wild-type or mock-infected extracts (Fig. 1A). The overall pattern and extent of synthesis was similar to that found after labelling of corresponding cultures *in vivo* (Fig. 1C), although a bias towards synthesis of larger proteins was evident in the cell-free products, presumably because much of the incorporation represents runoff synthesis (the completion of polypeptide chains started *in vivo*). Sensitivity to edeine, a specific inhibitor of polypeptide chain initiation, showed that incorporation was ≥75% dependent on *de novo* initiation in the d1331 system (Fig. 1B) and ~50% dependent in the mock and Ad-2 systems (data not shown). The low level of protein synthesis, coupled with the lack of polysomes in the d1331 extract (data not shown) and its dependence on reinitiation, provides circumstantial evidence for a defect in polypeptide chain initiation (see below and ref. 18).

Addition of polyadenylated RNA from Ad-2 infected cells to the d1331 extract increased incorporation slightly (Fig. 1B) but did not raise translational activity to a level comparable with the other extracts. Small amounts of Ad-2 hexon were synthe-

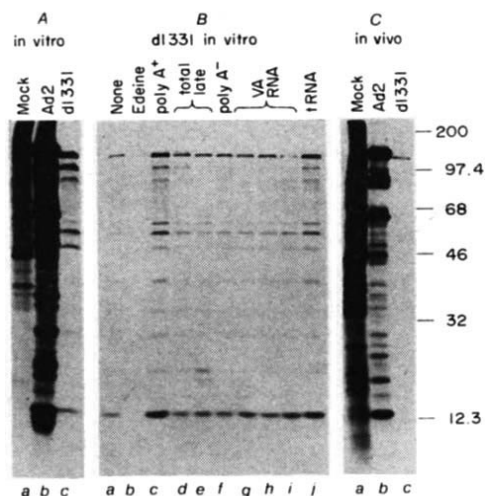


Fig. 1 Translational defect reproduced *in vitro*. **A**, Proteins synthesized *in vitro* by extracts of: *a*, mock-infected; *b*, wild-type Ad-2 infected; *c*, mutant d1331 infected HeLa cells. **B**, Translation products synthesized in a d1331 extract (*a*), supplemented with: *b*, 10 μ M edeine; *c*, 0.1 μ g oligo(dT)-selected mRNA from Ad-2 infected HeLa cells⁹ with 2 μ g carrier tRNA; *d*, *e*, 3.7 and 30 μ g cytoplasmic RNA from Ad-2 infected cells; *f*, 0.75 μ g poly(A)⁺ RNA from Ad-2-infected cells; *g*–*i*, 0.1, 0.3 and 1 μ g purified VA-RNA (ref. 4); *j*, 1 μ g calf liver tRNA (Boehringer Mannheim). **C**, Proteins synthesized *in vivo* by: *a*, mock-infected; *b*, Ad-2 infected; *c*, d1331-infected HeLa cells. At 24 h post-infection, cultures similar to those in **A** were labelled with ³⁵S-methionine for 2 h. Cells were collected and solubilized in gel sample buffer. The positions of size markers are shown (relative molecular mass $\times 10^{-3}$).

Methods. Extracts for protein synthesis were prepared from HeLa cells in suspension culture at 5×10^5 ml⁻¹. Cells were infected with virus at a multiplicity of 5 plaque-forming units per cell, and collected 24 h later. The cells were washed at 4 °C with phosphate-buffered saline (lacking Ca²⁺ and Mg²⁺) containing 2 mg ml⁻¹ glucose, suspended in two packed-cell volumes of 10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM dithiothreitol (DTT), allowed to swell for 10 min and disrupted in a Dounce B homogenizer. After centrifugation for 10 min at 4,000g and 15 min at 13,000g, the supernatants were stored in aliquots in liquid N₂. Before use in translation, extracts were thawed and clarified by centrifugation for 10 s at 10,000g. Standard reactions contained, in a volume of 25 μ l: 6.25 μ l HeLa cell extract supplemented with 15 mM HEPES pH 7.2, 100 mM KCl, 1.5 mM MgCl₂, 100 μ M spermidine, 1 mM ATP, 20 μ M GTP, 10 mM creatine phosphate, 50 μ g ml⁻¹ creatine kinase (Sigma), 1 mM DTT, 50 μ M each L-amino acid (methionine omitted), and 20 μ Ci ³⁵S-methionine (NEN). After incubation for 1 h at 30 °C, 5- μ l samples were taken for gel analysis and protein synthesis measurement. For gel electrophoresis, the samples were digested with RNase and proteins resolved in a 15% SDS-polyacrylamide gel³² were visualized by fluorography³³. For quantitation of ³⁵S-methionine incorporation, the samples were spotted on 3MM paper and the boiling trichloroacetic acid-stable radioactivity was determined in a scintillation counter. Further details will be reported elsewhere (P.A.R. and M.B.M., in preparation).

sized, migrating fractionally more slowly than its Ad-5 counterpart encoded by endogenous d1331 mRNA. In contrast to mock and Ad-2 infected extracts, the d1331 system was still unable to translate added Ad-2 mRNA efficiently after treatment with micrococcal nuclease to remove endogenous mRNA (Fig. 3). We conclude that the translational defect is not caused by lack of active mRNA or blockage of the ribosomes by defective mRNA.

VA-RNA does not act directly

Unfractionated late Ad-2 RNA and its poly(A)⁺ fraction failed to stimulate translation significantly in the d1331 system (Fig. 2B). As both preparations should contain VA-RNA₁, it seemed that VA-RNA might not be able to correct the defect in the

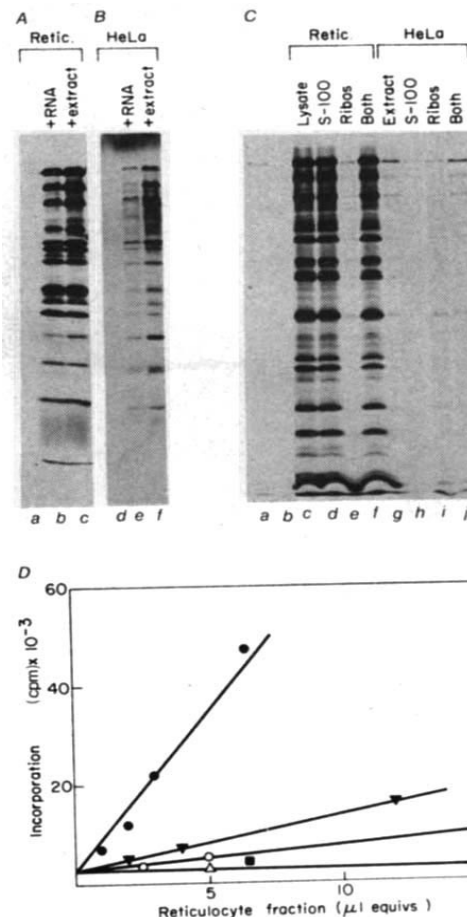


Fig. 2 Restoration of translational ability by extracts of reticulocytes and HeLa cells. Reactions containing micrococcal nuclease-treated extract from reticulocytes^{34,35} (**A**) or uninfected HeLa cells (**B**) were programmed with: *a*, *d*, no mRNA; *b*, *c*, RNA from 1 μ l d1331 extract (1.3 μ g); *e*, *f*, 1 μ l non-deproteinized d1331 extract. **C**, Reactions containing 6.25 μ l d1331 extract were supplemented with an equal volume of: *a*, d1331 extract; *b*, buffer; *c*–*f*, micrococcal nuclease-treated reticulocyte lysate, post-ribosomal supernatant (S100), ribosomes and a mixture of S100 and ribosomes; *g*–*j*, similar fractions from micrococcal nuclease-treated HeLa cell extract. **D**, The S100 (●), ribosomes (○), ribosome salt-wash (▼) and ribosomal subunit (△) fractions³¹ from micrococcal nuclease-treated reticulocyte lysate were titrated into incubations containing the d1331 extract as in **C**. ■, Addition of extra 6.25 μ l d1331 extract.

d1331 system. Accordingly, VA-RNA was purified from Ad-2-infected cells by gel electrophoresis as a mixture of the two species. It did not affect translation in the reticulocyte lysate or in mock or Ad-2 infected HeLa systems, demonstrating that it was free of inhibitory contaminants. Addition of purified VA-RNA to the d1331 system again failed to stimulate incorporation by more than 50% (Fig. 1B). No effect occurred over a concentration range from ~20 times less to 20 times greater than that found in the Ad-2 extract. Added VA-RNA also failed to increase translation of d1331 RNA in a micrococcal nuclease-treated HeLa or reticulocyte system (data not shown).

Furthermore, translational activity was not restored to a d1331 extract when it was supplemented with either mock- or Ad-2 infected cell extracts. On the contrary, the d1331 extract invariably depressed protein synthesis below the amount expected for the mock or Ad-2 extract alone (data not shown), implying that an inhibitor of protein synthesis accumulates in adenovirus-infected cells in the absence of VA-RNA₁. These results suggest an indirect effect of VA-RNA on translation, possibly in the modulation of the cytoplasmic level of the La antigen. Immunofluorescence demonstrates that this protein is in the nuclei of

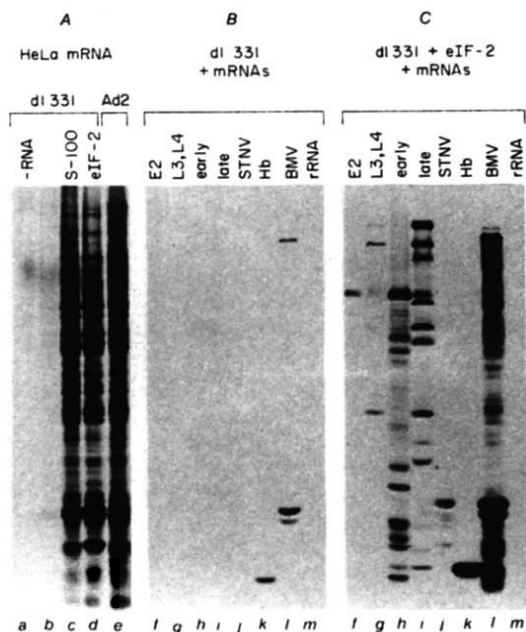


Fig. 3 eIF-2 allows many mRNAs to be translated in the d1331 system. **A**, HeLa RNA (10 µg) was translated in micrococcal nuclease-treated extracts of d1331 (**a-d**) or Ad-2 (**e**) infected cells. The d1331 incubations were supplemented with 6.25 µl reticulocyte S100 (**c**) or 1 µg eIF-2 (**d**). RNA was omitted from **a**. Reactions containing d1331 extract alone (**B**) or supplemented with 1 µg eIF-2 (**C**) were primed with: **f, g**, hybridization-selected³⁶ adenovirus RNA from early region E2 and late regions L3 and L4; **h, i**, unselected early³⁷ and late⁹ adenovirus RNA; **j**, satellite tobacco necrosis virus RNA; **k**, rabbit globin mRNA; **l**, brome mosaic virus RNA; **m**, *Escherichia coli* ribosomal RNA.

uninfected HeLa cells but moves into the cytoplasm late in adenovirus infection, possibly under the influence of VA-RNA (A. M. Francoeur and M.B.M., unpublished data). To determine whether the d1331-infected cell extract contained decreased concentrations of this protein, samples of the cell-free systems were transferred to nitrocellulose and probed with anti-La antibody using the immunoblotting technique^{11,19}. No significant differences in La antigen levels were observed (data not shown). Thus we have been unable to detect an effect of either VA-RNA or the La antigen on protein synthesis *in vitro*.

A stimulatory factor from reticulocytes

As expected from earlier results¹⁶, deproteinized RNA from the d1331 extract was almost as active as RNA isolated from the Ad-2 system in programming translation in the rabbit reticulocyte lysate system (data not shown). Significantly, we found that the viral mRNA was also translated when non-deproteinized d1331 extract was added to the reticulocyte system (Fig. 2A). Similar results were obtained using micrococcal nuclease-treated extracts of uninfected HeLa cells as the assay system, although with lower efficiency (Fig. 2B), indicating that the viral mRNA is present and intact in the d1331 extract and that it is not irreversibly sequestered or blocked.

This finding strongly suggests that translational activity might be restored to the d1331 system by the addition of components from either the reticulocyte lysate or HeLa cell extract. The HeLa extract indeed increased translation in the d1331 system; most of the stimulatory activity sedimented with the ribosomes but the maximal effect was only a two- to three-fold increase (Fig. 2C). The reticulocyte lysate, however, caused a marked stimulation of protein synthesis, restoring a large fraction of the translational potential of the endogenous mRNA. In this case, most of the activity was present in the post-ribosomal supernatant fraction (S100; Fig. 2C, D). A smaller portion (10–20%) of the stimulatory activity occurred in the ribosomes, it was liberated in the high-salt wash fraction and absent from the

Table 1 eIF-2 restores translation to d1331 extracts

Factor	Incorporation (c.p.m. × 10 ⁻³)			
	Expt 1		Expt 2	
	2 µl	6.25 µl	0.25 µl	1 µl
None		5.1		4.0
Extra d1331 extract (6.25 µl)		9.9		6.6
Reticulocyte S100 (6.25 µl)		ND		100.6
Initiation factors:				
eIF-2	96.4	90.0	34.0	67.5
eIF-3	6.3	5.2	4.2	4.1
eIF-4A	5.9	4.4	4.5	3.9
eIF-4B	ND	ND	5.1	6.2
eIF-4C	5.1	2.7	ND	ND
eIF-4D	9.7	11.4	ND	ND
eIF-4F	5.7	5.5	5.2	4.2
Elongation factors:				
EF-1	ND	ND	7.9	9.0
EF-2	6.4	6.0	6.8	4.0

Assays contained 6.25 µl d1331 extract with the purified factor indicated³¹. The factors eIF-2 to EF-1 were at the following concentrations, in the order listed: 1, 0.2, 0.8, 3, 0.5, 0.8, 0.7, 5 mg ml⁻¹ and EF-2 was at 5.8 and 2.2 mg ml⁻¹ in expts 1 and 2, respectively. ND, not determined.

Table 2 Specific initiation block in d1331 system

Factor added	Incorporation (c.p.m. × 10 ⁻³)		
	d1331	Mock	Ad-2
None	6.1	100.0	164.0
eIF-2 (2 µg)	82.2	85.2	144.0
eIF-2 (4 µg)	86.9	74.1	128.6
S100	100.5	105.9	198.4
S100 + eIF-2 (2 µg)	121.4	ND	ND

Reactions contained 6.25 µl HeLa cell extract and of reticulocyte post-ribosomal supernatant (S100) as indicated.

ribosomal subunits after dissociation by salt treatment (Fig. 2D). Such behaviour is characteristic of protein synthesis initiation factors and led us to test purified factors in the d1331 extract.

eIF-2 rescues translation *in vitro*

Of the seven purified initiation factors (IFs) tested, only eukaryotic initiation factor 2 (eIF-2) gave a response comparable with that obtained with the reticulocyte S100 (Table 1). Neither of the two elongation factors (EFs) was effective although EF-1 sometimes gave a small stimulation. We have also tested three less purified preparations that together comprise the ribosome salt-wash fraction (data not shown). One of these, containing eIF-3, eIF-4B, eIF-4F and EF-1, showed no stimulatory activity. The second subfraction contained eIF-4A, eIF-4D, EF-1 and EF-2 as well as mRNA and transfer RNA; it stimulated translation slightly but most of the activity was associated with RNA, shown by its sensitivity to micrococcal nuclease digestion. The third subfraction contained eIF-2, eIF-4C and eIF-5; it stimulated as effectively as eIF-2 alone. Although an effect of eIF-5 cannot be excluded at present, we conclude that eIF-2 is responsible for most, if not all, of the activity in the ribosome salt-wash fraction and that the translational defect in the d1331 extract is at the level of chain initiation.

The activity of eIF-2 was eliminated by edeine (data not shown), which we expected because this inhibitor acts at a site early in the initiation sequence. Similar results were obtained with the reticulocyte S100 fraction. We added the two preparations simultaneously to the d1331 extract to test whether the S100 action results entirely from eIF-2 (Table 2). The stimulations were not additive, consistent with an effect on the same reaction. The slight additional stimulation given by the S100 at saturating concentrations of eIF-2, however, was reproducible and suggested that the activities might not be fully congruent.

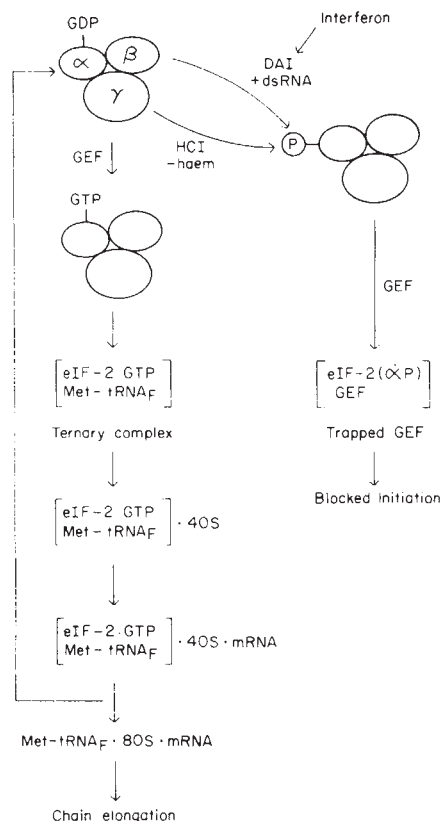


Fig. 4 The roles of eIF-2 and GEF in the initiation of translation. The α -subunit of eIF-2 can be phosphorylated by the haem-controlled inhibitor (HCl) or double-stranded RNA-activated inhibitor (DAI), leading to sequestration of GEF. Subsequently, eIF-2 molecules can participate no more than once in ternary complex formation.

As shown below, the S100 factor acts synergistically with eIF-2, which is not permanently inactivated in d1331-infected cells but held in check by an inhibitor.

Specificity of eIF-2

Late in adenovirus infection, protein synthesis is mainly viral, giving the impression that the translational block in d1331-infected cells may be specific for viral mRNAs (ref. 16). However, examination of the residual synthesis of host-cell proteins in infected cells made it probable that the effect of VA-RNA₁ applies indiscriminately to many mRNA species in the cell. This issue can be resolved *in vitro*. RNA from uninfected cells was translated poorly in the d1331 extracts (Fig. 3A). Addition of eIF-2 or the reticulocyte S100 fraction increased greatly the use of the HeLa mRNAs, restoring translation to a degree comparable with mock or Ad-2 extracts. Thus, the translational defect extends to most or all HeLa mRNAs. Furthermore, none of the following specific mRNAs was translated efficiently in the d1331 system unless eIF-2 (or reticulocyte S100) was added: early and late adenovirus-infected cell RNA and individual mRNAs hybrid selected from them; brome mosaic virus and satellite tobacco necrosis virus RNA, capped and uncapped plant viral RNAs, respectively and rabbit globin mRNA (Fig. 3B, C). The eIF-2 always enhanced translation 10–20-fold.

As our best HeLa cell extracts are only 20% as efficient as the reticulocyte lysate, we considered the possibility that the eIF-2 requirement might be a general feature of these systems, perhaps unrelated to the VA-RNA-specific defect of d1331. However, little or no enhancement of protein synthesis resulted from the supplementation of the mock and Ad-2 systems with eIF-2 or the S100 fraction, whereas a large stimulation was always observed in d1331 extracts (Table 2). These data indicate that this factor limits protein synthesis only in the mutant-

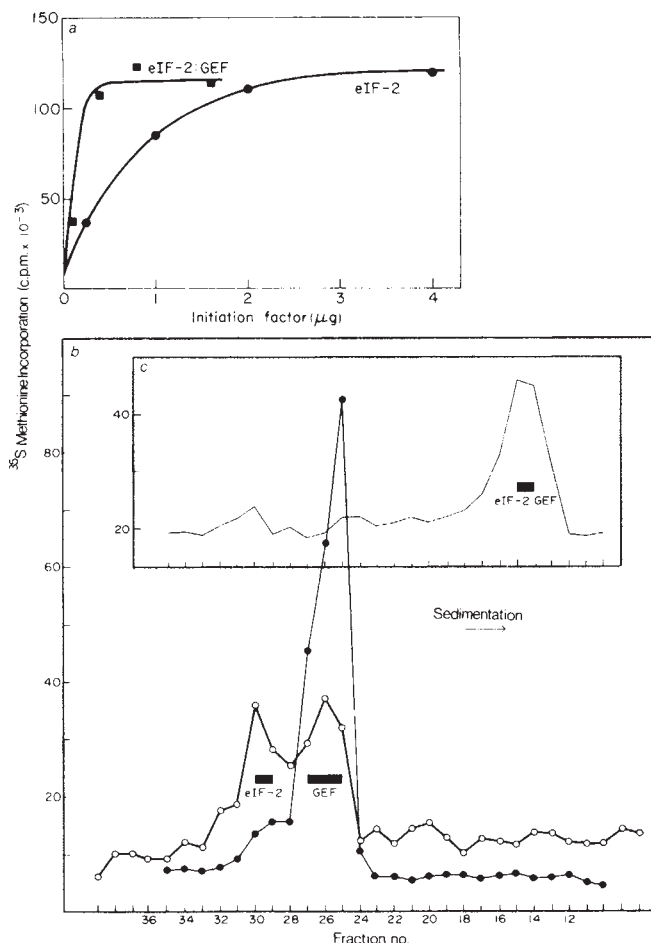


Fig. 5 Guanosine nucleotide exchange factor efficiently rescues protein synthesis. *a*, Essentially homogeneous preparations of eIF-2 (●) and eIF-2:GEF complex³⁸ (■) were titrated into 25- μ l reactions containing 6.25 μ l d1331 extract. GEF comprises about two-thirds of the complex by weight. *b*, Sucrose gradient of salt dissociated eIF-2:GEF complex. Fractions were assayed for d1331 rescue activity (●) and GEF activity (○). Rescue activity was monitored as in *a*. GEF was assayed by its ability to catalyse formation of ternary complex from eIF-2:GDP (ref. 38): free eIF-2 is also stimulatory. Bars mark positions of eIF-2 and GEF visualized by gel electrophoresis and silver staining of the gradient fractions. *c*, As *b*, except that the eIF-2:GEF complex was not dissociated. The plot shows rescue activity, which was coincident with GEF activity (data not shown). The bar marks the position of eIF-2 and GEF polypeptides visualized by staining.

Methods. 100 μ g eIF-2:GEF complex was incubated for 2 h at 0°C in 200 μ l 20 mM Tris-HCl, pH 7.5, 2.5 mM 2-mercaptoethanol and 500 mM or 100 mM KCl (for *b* and *c*, respectively). The factors were centrifuged for 22 h at 38,000 r.p.m. in a Beckman SW40 rotor through 12 ml linear gradients containing 10–30% (w/v) sucrose, 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 5% (v/v) glycerol and 500 mM or 100 mM KCl (for *b* and *c*, respectively). Fractions (0.3 ml) were collected, monitored spectroscopically and assayed.

infected cell system, and make it probable that the preferential use of viral mRNAs in adenovirus-infected cells is achieved independently of VA-RNA (possibly mediated by a viral protein^{20,21}).

Recycling factor

Our findings are most easily explained by the development in mutant-infected cells of a block in protein synthesis due to a failure of eIF-2 to recycle in its catalytic role (Fig. 4). In reticulocytes, such a block has been observed in several conditions, including haem deficiency and the presence of double-stranded RNA (reviewed in refs 22, 23). These stimuli activate specific protein kinases which phosphorylate the α -subunit of

eIF-2 and prevent the normal recharging of eIF-2:GDP to eIF-2:GTP, probably by trapping the guanosine nucleotide exchange (or recycling) factor GEF (refs 24–28), lack of which prevents eIF-2 from acting catalytically. The block in reticulocyte protein synthesis can be overcome experimentally by the addition of relatively large amounts of eIF-2 or smaller amounts of GEF.

Several observations argue that a similar mechanism operates in the d1331 extract. As shown above, the translational activity of d1331 extracts is enhanced greatly by the addition of pure eIF-2 and also by a factor found, like GEF, in the reticulocyte post-ribosomal supernatant. After partial purification, the supernatant factor contained little eIF-2 activity but substantial GEF activity (data not shown). GEF can be isolated as a complex with eIF-2; on a molar basis this complex was about 15-fold more effective than eIF-2 alone in rescuing translation (Fig. 5a). As expected, the activity co-sedimented with the eIF-2:GEF complex in native conditions (Fig. 5c), but when the complex was dissociated and its components resolved in a sucrose gradient, almost all the activity migrated with GEF (Fig. 5b). These results demonstrate a lesion in eIF-2 recycling; such a defect at this early stage of chain initiation (earlier than that previously thought¹⁸) would account for the observed lack of specificity for viral mRNAs²⁹.

Function of VA-RNA

We have shown that VA-RNA_i preserves the activity of eIF-2 in adenovirus-infected cells and that GEF has a pivotal role in

the process. Although there is no precedent for the involvement of small RNAs in regulating these factors, the appearance of a protein synthesis inhibitor in the mutant extracts offers an important clue. By analogy with the reticulocyte, a mechanism leading to the phosphorylation of eIF-2 seems most plausible. In the setting of viral infection, where double-stranded RNA is likely to be found, the double-stranded RNA-dependent protein kinase pathway may be involved. Despite its stable secondary structure^{5,7,8}, VA-RNA lacks the extended regions of perfect duplex necessary to trigger this kinase (S. Legon and M.B.M., unpublished data). In cells infected with wild-type virus, VA-RNA_i might form sufficiently stable secondary structures (either alone or with viral mRNA) to bind the kinase and block its activation by double-stranded RNA. Until the inhibitor is identified, however, alternative and possibly more direct interactions between VA-RNA and GEF cannot be excluded. The example of the 7S RNA-containing signal recognition particle, responsible for movement across the cell membrane of secreted proteins and regulating their synthesis³⁰, leads to the question of whether GEF might also possess an RNA subunit or cofactor. In this case VA-RNA would substitute for a cellular RNA whose production or stability is compromised by adenovirus infection.

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Note added in proof: We have shown recently that d1331 infected cells contain a protein kinase capable of phosphorylating eIF-2 and blocking initiation of transcription³⁹.

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LETTERS TO NATURE

Detection of OH radicals from IRAS sources

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Natural OH maser emission originates in the dusty circumstellar envelopes of highly evolved long-period variables and super-giant stars undergoing mass-loss. The OH spectra of such stars, at a microwave frequency of 1,612 MHz, are sharp double-peaked emission line profiles in which the two strongest features have a velocity separation of 5–60 km s⁻¹. Their far infrared colours are typical of sources with colour temperatures below 500 K. Most of

the 200 known OH/infrared stars were discovered in systematic OH-line surveys, at 1,612 MHz along the galactic plane^{1–4}, generally using small antennae and having a limiting sensitivity of ~1 Jy. But 40 well-studied sources were readily detected with the IRAS satellite⁵ at 12, 25 and 60 µm and were characterized by a limited range of infrared colours. A more efficient method for detecting new OH/infrared stars is to begin with IRAS source positions, selected for appropriate infrared colours, and using radio-line observations to confirm the OH properties. We demonstrate the validity of this approach here, using the Arecibo 305 m radio-telescope to confirm the 1,612 MHz line observations of sources in IRAS Circulars 8 and 9 (ref. 6); our present observations identify 21 new OH/infrared stars. The new sources have weaker 1,612 MHz fluxes, bluer (60–25) µm colours and a smaller mean separation between the principal emission peaks than previous samples.

The relatively cold distended atmospheres of highly-evolved stars allow dust grains to form and grow. These are slowly driven outwards by radiation pressure, and through collisions impart an outward velocity to the surrounding gas which expands and

Table 1 OH observations of IRAS sources at 1,612 MHz

Source (1)	$\langle V \rangle$ km s ⁻¹ (2)	ΔV km s ⁻¹ (3)	S_H mJy·km s ⁻¹ (4)	S_L mJy·km s ⁻¹ (5)	ΣS mJy·km s ⁻¹ (6)	(60-25) (7)	(25-12) (8)	(9)	1,667 mJy (10)
1806+091P08	-13.5	32.7	670	1,790	4,093	-1.160	-0.369	(2)	yes
1807+347P08	4.6	27.2	540	660	2,705	-1.132	-0.439	(2)	<10
1809+270P08	-4.5	13.6	64	10	516	-1.044	+0.106	(6)	yes
1812+051P08	86.9	13.9	6	14	n.d.	-0.794	-0.365	(3)	n.d.
1823+089P08	-8.1	19.1	120	60	610	-1.200	-0.416	(3)	n.d.
1823+218P08	75.7	11.8	20	17	181	-1.187	-0.413	(5)	n.d.
1825+078P08	93.7	16.4	730	1,200	2,805	-1.109	-0.316	(2)	n.d.
1912+172P09	n.d.	n.d.	<20	<20	n.d.	-0.717	-0.185		<15
1913+215P09	n.d.	n.d.	<20	<20	n.d.	-0.635	+0.127		n.d.
1917+199P09	46.4	23.6	1,060	470	2,680	-0.893	-0.194	(2)	n.d.
1920+156P09	59.6	25.4	900	430	2,877	-0.676	-0.134	(2)	<20
1920+210P09	55.4	27.2	1,390	1,310	5,809	-0.768	-0.013	(2)	
1922+302P09	n.d.	n.d.	<20	<20	n.d.	-0.701	+0.025		n.d.
1923+164P09	n.d.	n.d.	<20	<20	n.d.	-0.081	+0.542		n.d.
1923+167P09	n.d.	n.d.	<20	<20	n.d.	-0.481	+0.579		n.d.
1928+293P09	-38.2	30.9	360	710	4,866	-0.946	-0.190	(3)	yes
1930+141P09	n.d.	n.d.	<20	<20	n.d.	-0.671	+0.836		n.d.
1937+239P09	n.d.	n.d.	<20	<20	n.d.	-0.529	+0.292		<10
1938+152P09	5.0	32.7	400	780	4,085	-1.189	-0.407	(5)	<10
1938+154P09	62.0	26.3	20	50	101	-1.085	-0.354	(2)	yes
1944+228P09	-7.5	34.5	570	690	2,994	-0.747	-0.106	(4)	yes
1945+293P09	n.d.	n.d.	<20	<20	n.d.	-0.588	+0.367		<10
1945+172P09	-2.3	24.5	150	490	1,195	-1.012	-0.288	(3)	n.d.
1946+222P09	-30.8	28.1	30	170	393	-1.030	-0.200	(2)	n.d.
1947+240P09	n.d.	n.d.	<20	<20	n.d.	-0.688	+0.356		<10
1952+279P09	-16.3	n.d.	20	<20	n.d.	-0.133	+0.047	(1)	<30
1953+280P09	-31.1	22.7	490	1,200	2,617	-0.960	-0.169	(2)	
1954+305P09	n.d.	n.d.	<20	<20	n.d.	-0.805	-0.191		<20
1955+335P09	n.d.	n.d.	<20	<20	n.d.	-0.956	-0.324		<10
2005+185P09	n.d.	n.d.	<20	<20	n.d.	-0.917	-0.332		<20
2010+308P09	n.d.	n.d.	<20	<20	n.d.	-0.200	-0.106		<10
2013+286P09	-57.9	25.4	90	240	1,216	-0.970	-0.051	(2)	<10
2016+275P09	6.1	10.9	60	10	114	-0.416	-0.009	(2)	<10
2018+225P09	40.5	22.7	1,400	1,500	5,034	-1.045	-0.273	(2)	<10
WX Ser	6.3	14.5	1,570	1,290	5,915	n.d.	n.d.	(2)	n.d.

n.d., Not detected.

cools, forming stable molecules. At yet larger radii, dust opacity becomes small enough for the interstellar ultraviolet radiation field to penetrate and disrupt many molecules, while the gas density is also low enough for radicals like OH to be long-lived. The 1,612 MHz OH masing action takes place in the resulting thin, steadily expanding and almost spherically symmetric shell of gas; the component expanding directly towards us along the line of sight and the one expanding directly away from us are responsible for the characteristic double-peaked emission-line signature. Figure 1 is an example of a source with this structure at both 1,612 and 1,667 MHz.

The dusty cocoon surrounding a highly-evolved star absorbs much of its optical radiation. This is reradiated as a thermal spectrum in the far infrared at the lower temperature characterizing the dust. Olmon *et al.*⁵ found from IRAS observations, that this temperature is usually less than 500 K. Far infrared radiation at wavelengths of 35 and 53 μ m is also the source of the pumping power seen in the 1,612 MHz masers⁹. We therefore expect type II OH masers and far infrared sources, with appropriately low temperatures, to be highly correlated. Equipped for the first time by IRAS with an appropriate list of sources, we proceed to check the association of these infrared sources with 1,612 MHz masers.

In 1983, the Arecibo radio-telescope was equipped with a dual-polarization cooled 18-cm field effect transistor receiver, giving an effective system temperature of ~ 40 K at the zenith. Sources with a peak flux of 100 mJy are recognizable in one minute of integration; upper limits (3σ) of 20 mJy may be set with 15 min of integration time and a resolution of 4.9 kHz. This gives a 50-fold improvement in the limiting sensitivity compared with most earlier surveys. However, because of the IRAS positional accuracy of $\sim 45''$ and the Arecibo 3.2' half-power beam

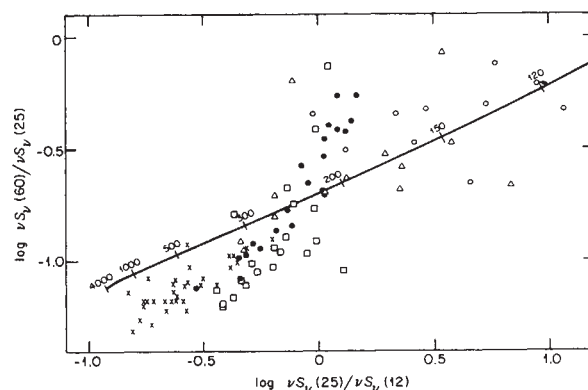


Fig. 1 Profiles at 1,612 and 1,667 MHz for a typical type II OH/infrared source, at a resolution of 4.88 kHz per channel. The strong narrow 1,612 MHz signal causes a slight ringing across the bandpass.

with a pointing accuracy of $\sim 10''$, we may sometimes miss a source.

We observed the IRAS positions of the 26 sources in Circular 9 falling within the Arecibo declination range, together with seven sources from Circular 8 having appropriate infrared colours. All objects were observed at 1,612 MHz in a frequency-switched mode, with an unsmoothed resolution of 4.88 kHz. A proportion of the objects have also been observed at 1,665 and 1,667 MHz. The flux scale is calibrated with sources taken from Bridle *et al.*⁷. Our data are summarized in Table 1, together with the infrared colours evaluated from the IRAS fluxes for

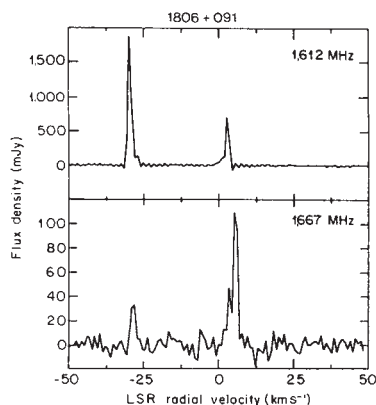


Fig. 2 Colour-colour diagram of the ratios between 12, 25 and 60 μm IRAS fluxes. Open squares, new detections; open triangles, undetected IRAS sources. Shown for comparison are previous data for OH/infrared stars, with large amplitude fluctuations in OH flux (filled circles) and small amplitude variations (open circles). Crosses, optical Mira variables, IRC and AFGL sources with OH emission. The line gives the locus of black bodies at various temperatures.

sources treated as 300 K black-body distributions⁸ (columns 7 and 8). The mean LSR (local standard of rest) velocity is given in column 2; the separation between the high and low velocity peaks in column 3, together with their peak fluxes at 4.88 kHz resolution in columns 4 and 5. Column 6 gives the integrated 1,612 MHz flux; column 9, the number of discrete features and column 10 an indication of the 1,667 MHz signal. These data are plotted in Fig. 2, together with the original data on Mira and OH/infrared stars⁵. To provide overlap with previous work, we include our data on WX Serpentis (Table 1).

Several points arise: (1) All detected OH fluxes are below the 1.7 Jy completeness limit of previous surveys⁴; six sources have peak fluxes < 100 mJy. (2) A 25–12 μm colour range of –0.5 to 0.1 typifies our detections and corresponds to colour temperatures of 200–400 K for the dust. (3) Our detections broaden the 60–25 μm colour range in the infrared colour-colour plot. As our sources are rather weaker than previous detections, this suggests that 1,612 MHz fluxes are being limited by the available 53- μm flux needed to maintain the basic 35- μm pump in a steady state equilibrium⁹. (4) The most extreme example in this respect is 1809+270, as its 1,612 MHz flux is much weaker than its signal at both 1,665 and 1,667 MHz, a feature not shown by any other source here. (5) The gap between sources with large amplitude swings in their OH signals and the low amplitude sources is accentuated⁵, as none of the four objects (1913+215, 1937+239, 1945+293 and 1947+240) observed to narrow the gap are detected. (6) Three objects not detected as OH emission sources (1923+167, 1922+302 and 1955+335) are in locations on the colour-colour plot suggesting that they should be detected; perhaps their data are affected by the 10% uncertainties associated with the provisional IRAS fluxes or by pointing errors. (7) The OH sources 1928+293, 1944+228 and 1806+091 are all type II with strong 1,667 MHz profiles but are undetected at the 50 mJy level at 1,665 MHz. The more intense of the two 1,612 MHz peak fluxes in each case is at the velocity of the weaker of the two 1,667 MHz peaks. Finally, (8) the sources examined at 1,667 MHz are usually the strongest infrared sources; most are not detected here at either 1,667 or 1,665 MHz.

The OH/infrared sources that we have confirmed generally have weaker 1,612 MHz fluxes than previously discovered detections. Most also have a bluer (60–25 μm) colour. As every IRAS source so far investigated with a 60–25 μm colour index < –1.0 is now confirmed as an OH/infrared star, the lower limit to the range of 60–25 μm colours associated with OH emission sources is yet to be determined. However, the mean ΔV for our sample at +23.1 (s.e.m. 1.6) km s^{-1} is significantly smaller than the 29.6 (s.e.m. 0.6) km s^{-1} found⁴ previously to characterize sources

away from the galactic centre. We therefore conclude that the use of IRAS colours to identify new OH/infrared stars changes their mean sample characteristics.

The infrared colours of type II OH sources are highly specific. For objects with a (25–12) μm colour in the range –0.5 to 0.1 our detection rate is 88%. This can be increased further by choosing sources within the data locus of Fig. 2. Away from the galactic centre, IRAS sources are rarely confused, so new OH/infrared stars may be identified with confidence by inspection of their far infrared colours. These studies are being continued at Arecibo.

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Plasma radiation during γ -ray bursts

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Most γ -ray bursts are thought to originate from or near the surface of neutron stars¹. Their spectra, however, have been measured only in the 30 keV–10 MeV energy range², with possible detection of some 1–12 keV X rays reported for several events³ (notably the Apollo event of 27 April 1972)⁴ at a level ~ 0.01 –0.02 of the γ -ray flux. Here I propose that γ -ray bursters may also strongly emit 0.05–1 keV XUV radiation simultaneously with the γ -rays, in the form of two narrow spectral lines, corresponding to the ω_e and $2\omega_e$ plasma radiation, where $\omega_e = (4\pi n_e e^2/m_e)^{1/2}$ is the electron plasma frequency of the γ -emitting (and pair annihilation) region of the burster. As estimates based on observed annihilation line intensities and other theoretical arguments typically give $n_e \geq 10^{24}$ – 10^{26} cm^{-3} (ref. 5), $h\omega_e \geq 37$ –373 eV, in the XUV range. If detected and their frequencies accurately measured with high time resolution, such radiation could be a valuable diagnostic tool for the emission region, as in the case of solar radio bursts⁶. Failure to detect them would also put strong constraints on the γ -emission models.

It has been demonstrated that enhanced ω_e and $2\omega_e$ radiation will be emitted by electron plasma oscillations (EPOs) in plasmas with steep density gradients⁷ or coexistent thermal and suprathermal electron populations^{8–10}. The ω_e line is due to EPO scattering from ion density fluctuations and the $2\omega_e$ line due to EPO-EPO (three-wave) scattering. In solar cases, the above conditions arise from shocks ascending the corona¹⁰. Such conditions are also likely to prevail in γ -burst emission regions. First, in many events, the simultaneous presence of hot optically-thin continuum emission and narrow line emission (probably the redshifted 511 keV line) and absorption (possibly cyclotron harmonics) are seen¹¹. These spectral features suggest the coexistence of two populations of electrons (the term 'electron' denotes both background electrons and e^+ pairs), one hot with $T_h \geq$ hundreds of keV and one cold with $T_c \leq 10$ keV. This interpretation is independent of whether the continuum emission mechanism is synchrotron⁵, bremsstrahlung⁴ or inverse Compton¹², and independent of the presence or absence of strong magnetic fields. Second, if strong magnetic field is present¹, then the rapid synchrotron cooling ($t_{\text{syn}} \approx 10^{-16}$ s

$(B/10^{12} \text{ G})^{-1}$) requires rapid heating or repopulation of the high-lying Landau levels. Coulomb collision with hot ions is inadequate¹. Only anomalous heating through EPOs ($\nu_e^{-1} \approx 10^{-17} \text{ s } n_{26}^{-1/2}$, $n_{26} \equiv n_e/10^{26} \text{ cm}^{-3}$) has a chance of matching such a cooling rate. Thus a strong field scenario would require the presence of copious amounts of EPOs. Moreover, the density gradient at a neutron star surface is probably steep. The combination of these two factors thus suggests efficient emission of plasma line radiations.

Because of the uncertainties of both the basic physics and characteristic parameters of the emission region, the estimates of the ω_e and $2\omega_e$ line intensities from γ -ray bursters given below are speculative, but they should encourage experimentalists to try to detect such radiations.

Consider first the case of weak fields, where the electron cyclotron frequency $\omega_{ce} \equiv eB/m_e c \ll \omega_e$ or $B \leq 3 \times 10^{10} \text{ G } n_{26}^{1/2}$, and the EPOs are generated by coexistent hot and cold isotropic electrons. Following Tidman¹⁰ we have

$$\begin{aligned} \epsilon_{\omega_e} &\approx 6 \times 10^{-25} n_e^{5/2} T_e^{-3/2} \left(\frac{V_h}{c} \right)^2 \alpha^{-4} \text{ erg cm}^{-3} \text{ s}^{-1} \quad (1) \\ \epsilon_{2\omega_e} &\approx 5 \times 10^{-25} n_e^{5/2} T_e^{-3/2} \left(\frac{V_h}{c} \right)^4 \alpha^{-3} \text{ erg cm}^{-3} \text{ s}^{-1} \end{aligned}$$

where n_e , T_e refer to the cold electrons:

$$\begin{aligned} \alpha &\equiv \left[2 \ln \left(\frac{n_h v_h}{(n_h + n_e) v_e} \right) \right]^{1/2} \\ v_h &\equiv \sqrt{\frac{T_h}{m_e}}, \quad v_e \equiv \sqrt{\frac{T_e}{m_e}} \end{aligned}$$

and n_h , T_h refer to the hot electrons. The above results are derived assuming $n_h \ll n_e$, $v_h \ll c$. But the errors in applying them to γ -burst regimes should not exceed the uncertainties in the parameters themselves. Denoting $n_{26} = n_e/10^{26} \text{ cm}^{-3}$; $T_{e5} \equiv T_e/5 \text{ keV}$ and $T_{h511} \equiv T_h/511 \text{ keV}$, we have

$$\epsilon_{\omega_e} \approx 6.15 \times 10^{28} n_{26}^{2.5} T_{e5}^{-1.5} T_{h511} \alpha^{-4} \text{ erg cm}^{-3} \text{ s}^{-1}$$

and

$$\epsilon_{2\omega_e} \approx 2.3 \times 10^{28} n_{26}^{2.5} T_{e5}^{-1.5} T_{h511}^2 \alpha^{-3} \text{ erg cm}^{-3} \text{ s}^{-1} \quad (2)$$

α is 0(1) for $n_h \sim n_e$ and n_{26} , T_{e5} , $T_{h511} \sim 1$. This should be compared with (assuming $n_h \sim n_e$) the inverse Compton emissivity $\epsilon_{ic} \approx 2 \times 10^{27} L_{038} n_{26} T_{h511} \text{ erg cm}^{-3} \text{ s}^{-1}$ ($L_{038} \equiv$ soft photon luminosity/ $10^{38} \text{ erg s}^{-1}$), optically-thin thermal bremsstrahlung emissivity $\epsilon_{tb} \approx 10^{30} Z n_{26} T_{h511}^{1/2} \text{ erg cm}^{-3} \text{ s}^{-1}$ ($Z =$ atomic number) and thermal synchrotron emissivity $\epsilon_{ts} \approx 3.2 \times 10^{31} n_{26} B_{10}^2 T_{h511}^2 \text{ erg cm}^{-3} \text{ s}^{-1}$ ($B_{10} = B/10^{10} \text{ G}$, see ref. 1). Hence, if both line and γ emissions are optically thin and the EPO region coincides with the γ emission region, for $T_h \approx 511 \text{ keV}$ the line radiation flux at Earth would be $\sim 10 L_{038}^{-1}$ of the inverse Compton γ -flux, $\sim 10^{-2} n_{26}^{-1} Z^{-1}$ of the thermal bremsstrahlung γ flux and $\sim 10^{-3} B_{10}^{-2}$ of the thermal synchrotron γ flux. These ratios become invalid if the EPO region becomes thick enough so that the ω_e and $2\omega_e$ lines are self-absorbed. In that case, we expect their intensities to be limited by the Rayleigh-Jeans distribution of the hot electron temperature. For $T_h = 511 \text{ keV}$, $F_{\nu}^{\text{RJ}} \approx 10^{25} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1} n_{26}^{3/2}$ and the total line flux depends on the linewidth. The linewidth from electron Doppler would be quite narrow: $\Delta\nu/\nu_e \sim (T_e/m_e c^2)^{1/2} \sim 0.1$. But the main broadening may be due to density gradients. For solar plasma lines this is typically $\Delta\nu/\nu_e \approx 0.4$ (ref. 8). Assuming a linewidth of $\Delta\nu \approx 100 \text{ eV}$, the Rayleigh-Jeans-limited line flux would be $F^{\text{RJ}} \approx 10^{34} \text{ erg km}^{-2} \text{ s}^{-1} n_{26}^{3/2}$. More importantly, the lines are discernable only if they are not overwhelmed by the low-frequency emissions of the hot plasma or other lower temperature continuum emissions from a larger area, such as the neutron star magnetosphere. These uncertainties can only be settled by observations. We also expect the frequency to drift with time as the emission region evolves. So the time-integrated profile (for example, tens of milliseconds) could be smeared

but probably still resolvable. Although this frequency range is highly opaque in the galactic equator (visibility $< 100 \text{ pc}$), there are known transparent windows at high latitudes, including the positions of the recurrent sources^{13,14}. We are thus optimistic that a nontrivial number of galactic bursters ($\leq 10 \text{ kpc}$) may be detectable since the sources apparently have an isotropic distribution.

Next we consider the case of strong magnetic fields ($B \gg 3 \times 10^{10} \text{ G } n_{26}^{1/2}$) where the purely longitudinal EPOS can only propagate along field lines. Because the ion cyclotron and plasma frequencies are both $\ll \omega_e$ even at $B = 10^{12} \text{ G}$, the ω_e radiation is, to the first order, unaffected by the B -field. But we expect the $2\omega_e$ radiation to be strongly inhibited since colinear EPO-EPO scattering cannot directly produce transverse electromagnetic waves and we have to rely on higher order processes such as scattering of transverse ω_e radiation with EPOS and so on. Of course, the EPOS can also scatter from the other collective oscillations not propagating parallel to $\vec{B}$ (for example, lower hybrid resonance). But all these waves have frequencies $\approx \omega_{ce}$ since $\omega_{ce} \gg \omega_e$. So the resultant electromagnetic radiation will have a characteristic frequency near the electron cyclotron frequency, making them indistinguishable from ordinary cyclotron harmonic emission (except perhaps for their coherence). Hence the ratio of ω_e to $2\omega_e$ line intensity may be a useful probe of the B -field strength.

More speculatively, suppose that the hot electrons in the strong field case are indeed heated by copious amount of externally induced EPOS. Then the EPO energy flux must be comparable with the hot electron energy flux. Unlike the previous case where the EPOS are secondary emissions by the hot electrons, here they are the primary energy source of the γ flux and probably emit enhanced ω_e radiations. Specifically, in the case where the EPOS couple to ω_e radiation through a steep density gradient of scale height L ($> 2\pi c/\omega_e$), using Boyd and Sander-son¹⁰ the ω_e emissivity is estimated at

$$\epsilon_{\omega_e} \approx \frac{2\pi c^2}{3\omega_e} \frac{|E_0 \sin \theta|^2}{L^2} \quad (3)$$

where E_0 is the amplitude of the oscillating plasma field and θ is the angle between $\vec{\nabla} n$ and $\vec{E}_0$. Assuming $(\sin^2 \theta) \approx 1/2$, $cE_0^2/8\pi = F_\gamma$ (γ flux) and ω_e emission region thickness $\sim L$, we have for the total ω_e flux

$$F_{\omega_e} \sim \frac{8\pi^2}{3} \frac{c}{\omega_e L} F_\gamma \quad (4)$$

In this case, the Rayleigh-Jeans limit is no longer valid since the EPOs could be strongly coherent. Finally, we note that in the scenario where the hot electrons are produced by resonant absorption of intense relativistic Alfvén waves^{15,16}, the reflected ω_e radiation would be comparable with the absorbed flux $F_0 \sim 10^{30} \text{ erg cm}^{-2} \text{ s}^{-1}$ (see ref. 5) and the total plasma line flux could be comparable to the γ flux itself.

Thus, if we believe that γ bursts originate from dense plasmas at or near the surface of a neutron star, then they should also emit significant level of plasma line radiations at 0.05–1 keV. The minimum luminosity in these lines would be $\sim 10^{-6}$ – 10^{-7} of the γ -luminosity independent of source distance if the γ rays are synchrotron in a 10^{12} G field but the electrons not heated collisionlessly. The line luminosity could go as high as the γ luminosity itself if the hot electrons are heated by resonant absorption of intense magnetic reconnection wave fluxes. It could even exceed the γ luminosity if the γ rays are due to inverse Compton. Detection of the line radiation would provide clean diagnostics of the emission region (such values as n_e , T_e , B and ∇n_e), as well as discriminations between different mechanism of γ emission and electron heating.

The far-reaching implications of the possible discovery of such line radiations require the development of a comprehensive experimental programme to keep a close eye on the high galactic latitude windows where one can see through the galaxy in these frequencies. In particular, the N49 (Large Magellenic Cloud)

region which contains the known recurrent source¹³ that may be periodic¹⁷, should be kept under surveillance in the XUV at least near phase 0. Hopefully, future high-energy explorer development (such as High Energy Transient Explorer and Extreme Ultraviolet Explorer) would keep these observations in mind.

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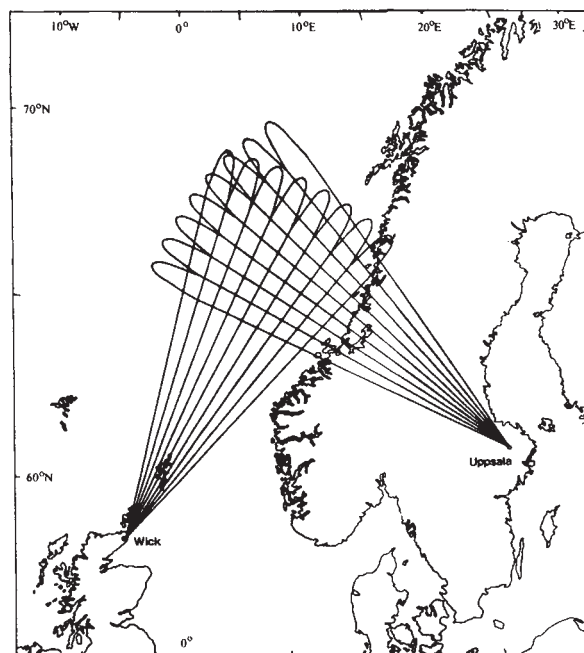


Fig. 1 Beam geometry of the Wick and Uppsala SABRE radars, indicating their common viewing area.

Mean auroral E-region plasma convection patterns measured by SABRE

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Large-scale flows of convective plasma in the Earth's magnetosphere, driven by the external influence of the solar wind, have long been known. The electric fields associated with this convection are mapped along approximately-equipotential field lines to low altitudes, resulting in strong ionospheric flows. Considerable controversy surrounds the detailed pattern of the convective flow in the ionosphere, its dependence on geomagnetic and interplanetary conditions and the resulting magnetospheric topology. Several observations^{1,2} suggest that this pattern rotates towards earlier local times during magnetically-disturbed periods, but empirically-based modelling failed to detect such an effect and even suggested the counter rotation, towards later local times³. Since April 1982, a new radar auroral backscatter system, SABRE (Sweden and Britain Radar auroral Experiment) has been in operation in northern Europe⁴, which allows estimates to be made of plasma convection in the auroral E region over a large area ($\sim 200,000 \text{ km}^2$, $L = 4-6$) with high spatial and temporal resolution. Here we present the flow patterns averaged over this period of radar operation for different levels of magnetic activity, revealing a definite rotation of the whole convection pattern towards earlier local times with increasing activity.

The radar auroral technique used in the SABRE system is similar to that of the earlier STARE (Scandinavian Twin Auroral Radar Experiment) system⁵ and comprises two multi-beam radar installations located respectively at Wick in Scotland and Uppsala in Sweden (Fig. 1). The radar systems are sensitive to electrostatic plasma waves (irregularities) of $\sim 1 \text{ m}$ wavelength produced in the auroral E layer by the two-stream and gradient-

drift plasma instabilities. These irregularities propagate approximately perpendicular to the geomagnetic field and measurement of their phase velocity in two independent directions (from the Doppler shift of the backscattered-radar signal) yields a close estimate of the electron-drift velocity within the observing area^{6,7}.

A unique advantage of the SABRE/STARE technique is the high spatial and temporal resolution possible ($20 \times 20 \text{ km}^2$ and 20 s respectively) compared with other methods. Furthermore, these systems provide continuous data coverage when conditions permit (see below), with high reliability and moderate cost. But there are also several disadvantages: first, plasma irregularities must be present to provide radar backscatter and therefore measurements are not possible during 'quiet' times, when the electric field is below the threshold of $\sim 15-20 \text{ mV m}^{-1}$ necessary to excite these waves; second, comparison with results from the European Incoherent Scatter facility (EISCAT) indicates that SABRE and STARE progressively underestimate the higher flow speeds ($\geq 600 \text{ m s}^{-1}$), although the flow directions are accurate⁸.

The most important factor governing the morphology and properties of the Earth's magnetosphere and its plasma populations is the convection of flux tubes and associated plasma, driven by the solar wind. The resulting two-cell pattern of plasma circulation at high latitudes in the ionosphere⁹⁻¹¹ is illustrated in Fig. 2. The main features of this circulation pattern are: (1) the strong zonal flow components near dawn and dusk, corresponding to the morning and evening auroral electrojet currents and (2) the reversals near magnetic noon and midnight. The morning discontinuity is located in the region of the magnetospheric cleft where the configuration of the geomagnetic field is such that magnetosheath particles can penetrate directly to ionospheric altitudes. It is therefore a very dynamic region for which an example of the flow pattern observed by SABRE near the morning reversal has been reported recently¹². The evening (or Harang) discontinuity usually extends to lower geomagnetic latitudes than the morning reversal. Observation and theory suggest that the relative shape and size of the convection cells varies considerably both with magnetic activity and the polarity of the interplanetary magnetic field (IMF)^{2,13,14}. In particular, STARE observations² indicate that as magnetic activity

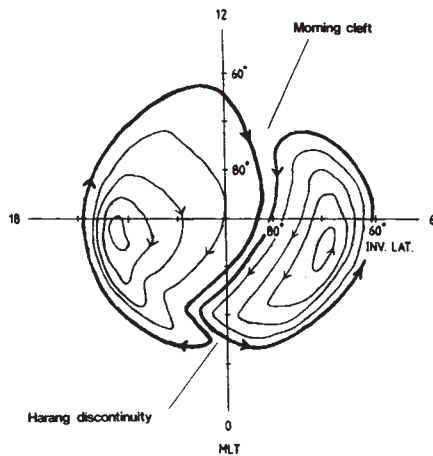


Fig. 2 Schematic illustration of the average high-latitude convection pattern¹⁷.

increases, (1) the electric-field magnitude increases, (2) the average location of the field maximum moves equatorwards, (3) the morning cell expands and (4) the whole convection pattern rotates towards earlier local times.

The causes of magnetospheric convection have been discussed in terms of two main theories, one in which closed magnetospheric-flux tubes are transported from the day side to the night side in a boundary layer around the flanks of the magnetosphere by a quasi-viscous process occurring at the magnetopause¹⁵ and the other in which open flux tubes are transported over the poles of the Earth after reconnection with the IMF on the day side magnetopause¹⁶. It has also been suggested that these processes coexist, with the 'viscous' process contributing a small but sometimes significant 10–30 keV¹⁷ to the usual cross-magnetosphere potential of 40–100 kV.

Averaged measurements of the convection pattern have been reported previously by incoherent scatter radars^{10,18,19}; the present results, however, represent the first study of this kind using the so-called 'coherent' radar technique over such an extended period, with a much larger data set of ~380 days compared with typically 5–10 days of incoherent scatter data.

Figure 3 illustrates the plasma convection pattern observed by SABRE, averaged over all conditions; it is a composite of available measurements. The main features of the pattern are identified readily: strong electrojets involving sunward convection in the dawn and dusk sectors and the morning and evening discontinuities at around 09.00 UT and 21.00 UT respectively. The evening reversal is slightly later (by ~1 h) at lower latitudes, consistent with the average configuration of the flow pattern at these latitudes (Fig. 2).

To investigate the variation of the plasma convection pattern with magnetic activity, the average convection velocities at 64°N were calculated for each value of K_p . The results of this analysis are plotted in the same format as Fig. 3, but with the ordinate representing K_p instead of latitude, in Fig. 4, the most striking feature of which is the shift of the Harang discontinuity to earlier local times, with increasing activity, from ~21.30 UT at $K_p=2$ to ~19.00 UT at $K_p=6$. At higher K_p , flows become more irregular. This is expected, particularly as for these conditions the data are averaged over fewer days and hence individual perturbations will have a greater effect. On the other hand, the flows in the region of the morning reversal are weak under all but the most active conditions.

Our results extend previous STARE observations of the mean pattern of convection in the ionosphere² and represent the first such study using the auroral radar technique on a time-scale of years. We have confirmed that a basic two-cell convection pattern exists in the high-latitude ionosphere and that this pattern has a clear apparent rotation towards earlier local times with increasing magnetic activity. This highlights the problems inher-

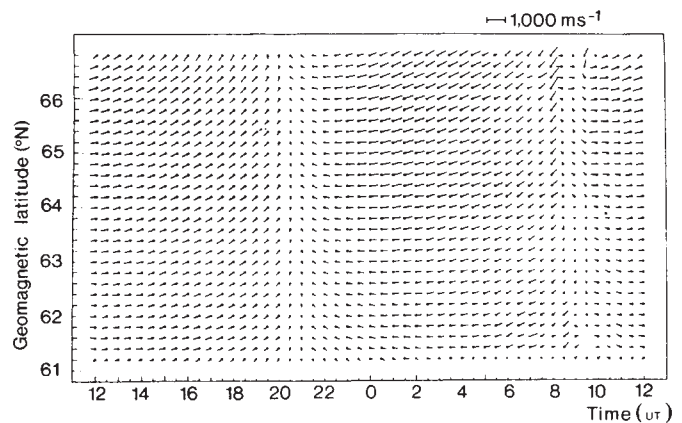


Fig. 3 Average convection velocities measured by SABRE (magnetic midnight is approximately 22.30 UT). The data are presented as a function of geomagnetic latitude and universal time, averaged over 4° of longitude. The magnitude and direction of the convection flow are represented by the length and direction of the vector plotted at each grid point, with north towards the top of the figure. Flow measurements were only included in the statistics if the corresponding backscatter signal/noise ratio was >3 dB; the number of data values therefore varies with latitude and time, fewer points being available at lower latitudes and during the morning hours.

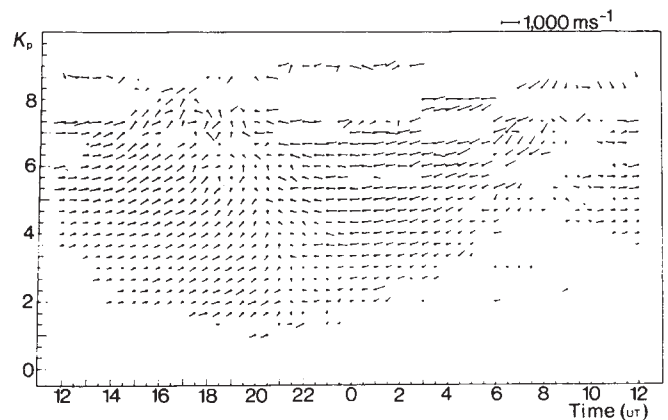


Fig. 4 Illustrating the variation of K_p of the convection velocities measured by SABRE at 64°N. Gaps in the data are because of either an absence of backscatter (low K_p values), or non-occurrence (high K_p values).

ent in interpreting data from only one location, as the apparent rotation of the convection pattern to earlier times with increasing activity may result from a bulk rotation of the whole convection pattern, a displacement of the pattern with respect to the pole, or enhanced asymmetry of the morning and evening cells. However, there are now several radar facilities in the Northern Hemisphere and collaborative efforts to monitor simultaneously the plasma convection pattern at different geomagnetic longitudes should be made to resolve this ambiguity. Future work should also resolve whether this rotation is a repeatable regular feature of strong magnetosphere-IMF coupling, or a transient effect associated with substorm-related ionospheric current and conductivity enhancements. Spacecraft measurements of the IMF polarity will be used to examine this question. The British radar is operated by Leicester University; the German radar is operated by MPAE in cooperation with the Uppsala Ionospheric Observatory.

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Downward flux of atmospheric 63- μm emission from atomic oxygen at balloon altitudes

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The 63- μm emission line from the ground electronic state fine-structure transition ($^3\text{P}_1$ - $^3\text{P}_2$) of atomic oxygen, first suggested as a major source of thermospheric cooling by Bates¹ and subsequently discussed theoretically^{2,3}, has been measured over a range of thermospheric altitudes⁴⁻⁶. The rocket measurements showed that the downward intensity remained essentially constant between 85 and 100 km, as expected for an optically-thick emitting region. As a result, OI emission is now thought to be less important as a source of atmospheric cooling than upward radiation from the 5.3 μm band of NO (ref. 7). Nevertheless, measurements of the intensity distribution of OI emission in the lower thermosphere should help to discriminate between theoretical models⁸ and, in particular, address the appropriateness of 'local thermodynamic equilibrium' at these altitudes. We report here high spectral resolution measurements of OI emission at 30 km. The downward OI flux is measured to be $(2.4 \pm 0.5) \times 10^{-5} \text{ W m}^{-2} \text{ sr}^{-1}$, somewhat larger than expected on the basis of previous rocket measurements or theoretical predictions. Furthermore, this value is found to be independent of zenith angle.

The observations reported here were made on a nighttime balloon flight from the National Scientific Balloon Facility at Palestine, Texas with the University College London-European Space Agency astronomical spectrometer⁹. Table 1 lists the relevant system parameters. The flight objective was to obtain infrared spectra from a number of astronomical sources. For this, background spectra—the basis of the present analysis—covering a range of zenith angles corresponding to air-mass values of 1.27–>20 (one air mass refers to the integrated path of the zenith at this altitude) were required to eliminate any contribution to the observed spectra from the Earth's atmosphere.

Interferograms obtained with the Fourier spectrometer were processed using standard techniques. The wavelength scale of the resulting spectra was determined from the positions of several water-vapour lines. Flux calibration was determined from spectra obtained with an in-flight black body operating at

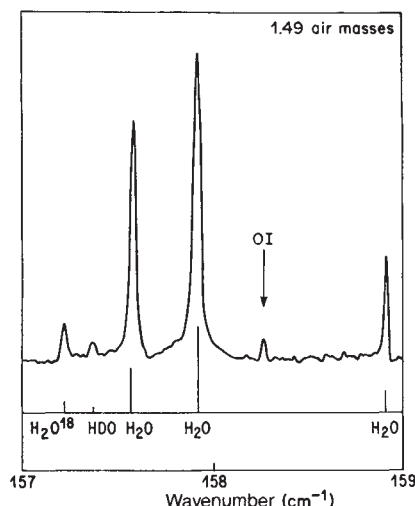


Fig. 1 Background spectrum at 157–159 cm^{-1} , identifying the OI line at 158.2686 cm^{-1} , along with neighbouring isotopic H_2O lines.

two stabilized temperatures. Details of the data processing and calibration procedures will be published elsewhere¹⁰.

A short section of an atmospheric spectrum between 157 and 159 cm^{-1} , taken at an air mass of 1.49, is shown in Fig. 1. The OI line is clearly identified, together with several neighbouring isotopic H_2O lines¹¹. The position of the OI line is determined to be $158.269 \pm 0.003 \text{ cm}^{-1}$, which is in excellent agreement with the inherently-more-precise laser magnetic resonance (LMR) measurement of $158.26862(7) \text{ cm}^{-1}$ (ref. 12), when the reported wavenumber is corrected for an error in the assignment of the laser wavelength used in the LMR experiment (R. M. Evenson, personal communication).

Measurement of the OI line and adjacent stratospheric H_2O lines, over a range of zenith angles equivalent to air-mass values of 1.27–>20, have revealed an interesting feature, shown in Fig. 2: the OI line intensity remains constant as the zenith angle and thus the equivalent air mass changes over the full range of measured values, whereas neighbouring stratospheric H_2O lines show the expected increase in line intensity as a function of air mass. This feature is more graphically illustrated in the plot of effective equivalent width as a function of the square root of the air mass, shown in Fig. 3. Also shown (Fig. 3) for comparison is a similar plot for a nearby stratospheric H_2O line of comparable strength, at low air mass, exhibiting the expected linear dependence on $(\text{air mass})^{1/2}$ of an emission line whose profile is dominated by pressure broadening in the strong regime of its curve of growth¹³. The constancy of the OI line intensity stems

Table 1 System parameters for night-time balloon flight from Palestine in Texas

Flight details	
Date	22 October 1980
Observation period	19:00–05:00 local time
Location	Palestine, Texas (32° N)
Altitude	30 km
Telescope	
Size of primary mirror	60 cm
Field of view	1.24 arc min
Calibration	in-flight black-body
Spectrometer	
Type	Fourier transform
Spectral range	100–200 cm^{-1}
Resolution	0.01 cm^{-1}
Time per interferogram	120 s

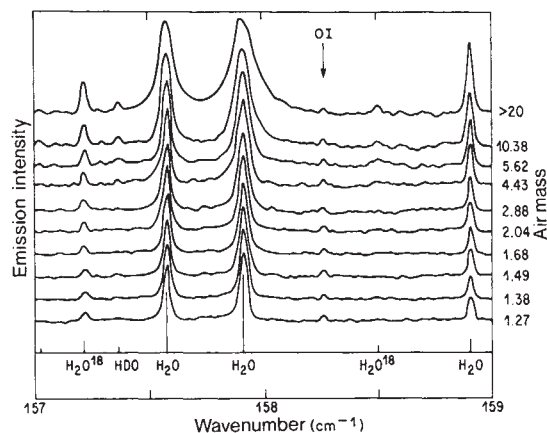


Fig. 2 Ten background spectra at various air-mass values, showing the increase in emission of the isotopic H₂O lines, and the constant intensity of the OI line.

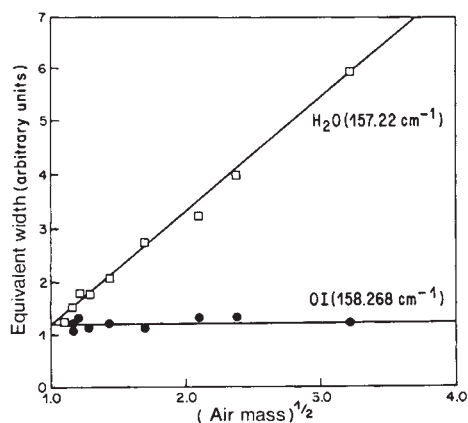


Fig. 3 Curve of growth for the OI line (158.2686 cm⁻¹) and an H₂O line (157.22 cm⁻¹) using data from Fig. 2. The error in the equivalent-width values is represented by the size of the plotted symbols. (See text for details.)

from the fact that its profile is dominated by Doppler broadening. As the temperature in the source region to optical depths greater than unity (at 95 km at the line centre²) remains approximately constant, the line intensity for a Doppler-broadened line is not expected to vary with air mass—a regime discussed in standard texts on radiative transfer, for example in Thorne¹⁴.

The calibrated OI line intensity, as a mean of all the spectra, is $2.4 \times 10^{-5} \text{ W m}^{-2} \text{ sr}^{-1}$, to an estimated accuracy of 20%. This value is significantly higher than those obtained from previous rocket flights ($1.2 \times 10^{-5} \text{ W m}^{-2} \text{ sr}^{-1}$ at 90 km (ref. 15), $7.2 \times 10^{-6} \text{ W m}^{-2} \text{ sr}^{-1}$ between 85 and 100 km during an auroral display⁵, and $4.5 \times 10^{-6} \text{ W m}^{-2} \text{ sr}^{-1}$ at 120 km (ref. 4)). A theoretical prediction³ of downward zenith intensity, of $9.4 \times 10^{-6} \text{ W m}^{-2} \text{ sr}^{-1}$ at 50 km, also is lower than the measured value, whereas model calculations⁸, allowing for a change in OI concentration by a factor of 4, predict values of $1.0\text{--}1.7 \times 10^{-5} \text{ W m}^{-2} \text{ sr}^{-1}$ at 80 km, in closer agreement with the observed value. The OI intensity in the optically-thick regime depends on atomic oxygen content (which can vary over an order of magnitude¹⁶) and temperature, which is also variable. The lack of agreement among all these values is therefore not surprising. Solar-activity effects on the thermopause temperature and auroral activity are not expected to play a significant role in determining the downward OI flux measured in the stratosphere, as the source function is believed to peak in the lower thermosphere.

In conclusion, measurements of atmospheric OI emission obtained with a balloon-borne telescope and a high resolution Fourier transform spectrometer verify the corrected LMR wavelength of $63.18372 \mu\text{m}$ for this transition and give a downward integrated line intensity which does not vary by more than 5% over the prescribed range of zenith angle.

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Charnockite formation at Ponmudi in southern India

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Charnockites, constituents of most Precambrian high-grade terranes^{1,2}, are essential for understanding the evolution of the early continental crust. The arrested development of charnockite in shear veins at Kabbaldurga³, in the state of Karnataka in India, suggests flow of CO₂-rich water-deficient fluids through deep-seated rocks as a mechanism of granulite grade metamorphism^{4,5}. We report here an occurrence of arrested charnockite formation well south of Kabbaldurga, in the khondalite belt of southern Kerala, where rocks with amphibolite facies give way to the vast southern India–Sri Lanka charnockite terrain, indicating that metamorphism due to CO₂-rich fluids (carbonic metamorphism) may have operated over a large area in southern India. If such localities prove to be widespread, the present level of exposure throughout much of the high-grade terrain probably does not extend far beyond an isofacial surface marking the boundary between upper and lower crust in the late Archaean.

The khondalite belt of Kerala and Tamil Nadu (Fig. 1) is a dominant supracrustal sequence consisting of metapelites with granulite facies (khondalites) and subsidiary bands of quartzites, graphitic paragneisses, charnockites and carbonates with lenses of basic and ultrabasic granulites. The age of the belt is not well-established; only single whole-rock Rb/Sr ages are reported, ranging from 2,155 Myr to 3,070 Myr⁶. Although these dates indicate an Archaean age, we cannot exclude a Proterozoic age. The exposures described here occur in a quarry 1 km east of the hill resort of Ponmudi, about 65 km north of Trivandrum in the northern portion of the khondalite belt (Fig. 1). The quarry contains roughly equal proportions of a grey-white biotite/garnet/plagioclase/K-feldspar/quartz/graphite gneiss and coarse-grained dark-green charnockite. The gneiss has both a well-developed foliation and a composition banding trend along the direction N70°W, coinciding with the regional foliation which is cut by patches of charnockite 0.1–1 m in width (Fig. 2). In places the charnockite is concentrated in sheared limbs and hinges of small folds; warping of gneissic foliation can be seen at the edges of the charnockitic patches. The charnockite appears to be concentrated along two directions, N50°E

and E-W, which could be a conjugate set of shear planes. The field relationships indicate clearly that the charnockite was formed after the gneiss; in this respect the exposures at Ponmudi resemble those at Kabbaldurga, 500 km to the north. However, a major difference between the two localities is that the latter is heavily infused with granite veins whereas the former shows none. In this respect, and because of the lack of any marked retrogressive recrystallization, the Ponmudi quarry is a remarkably simple and clear case of charnockite formation.

Petrographic examination demonstrates the sharp decline in the abundance of biotite in charnockites. Red-brown biotite is common and uniformly disseminated in the grey gneiss but is rare in the charnockite, where it occurs mainly as inclusions within garnet and orthopyroxene. Plagioclase, quartz and K-feldspar occur in both types of rock. However, crystals are usually much larger in the charnockite, where they range up to 1 cm across, compared with typical 1–2 mm grains in the gneiss. Graphite is abundant in both gneiss and charnockite, especially so near their interface, indicating mobility during the recrystallization. Garnet is also common in both rock types and appears to have undergone little or no recrystallization during the formation of the charnockite. Ilmenite and apatite are present in both rocks, but magnetite is absent.

Rock analyses were performed by X-ray fluorescence analysis on homogenized powders of fist-sized chunks taken about 10 cm apart from opposite sides of a charnockite–gneiss margin in two different specimens (Table 1). Mineral analyses on the same rocks used an ARL-EMX microprobe, with an energy-dispersive detection and data reduction scheme similar to that of Reed and Ware (ref. 7; see Table 1). Our rock analyses of the two grey gneiss–charnockite pairs show that the transition from gneiss to charnockite is remarkably isochemical, similar to the transformation at Kabbaldurga. Garnet, plagioclase and biotite have almost identical compositions on both sides of the transformation boundary. Orthopyroxene is $Mg_{0.34}Fe_{0.66}SiO_3$, somewhat more Fe-rich than at Kabbaldurga. Biotite is very high in TiO_2 , marking it as a granulite facies variety, stable at high temperatures and low H_2O pressures⁸. The experimental garnet–biotite K_D^{Fe-Mg} thermometer⁹ reads 700 °C at 6 kbar, probably too low because of a perturbing effect of the high Al and Ti contents of biotite¹⁰. A garnet–orthopyroxene experimental K_D^{Fe-Mg} thermometer¹¹ reads 820 °C, with an uncertainty of ± 60 °C. A plausible upper-temperature limit may be given by the vapour-absent melting reaction of biotite and quartz to K-feldspar, orthopyroxene and liquid at 810 °C at ~ 5 kbar¹², thus a reasonable temperature may be 760 ± 40 °C. A garnet–plagioclase–orthopyroxene–quartz geobarometer reads either¹³ 6.1 kbar at 760 °C or¹⁴ 6.0 kbar at the same temperature. Thus,

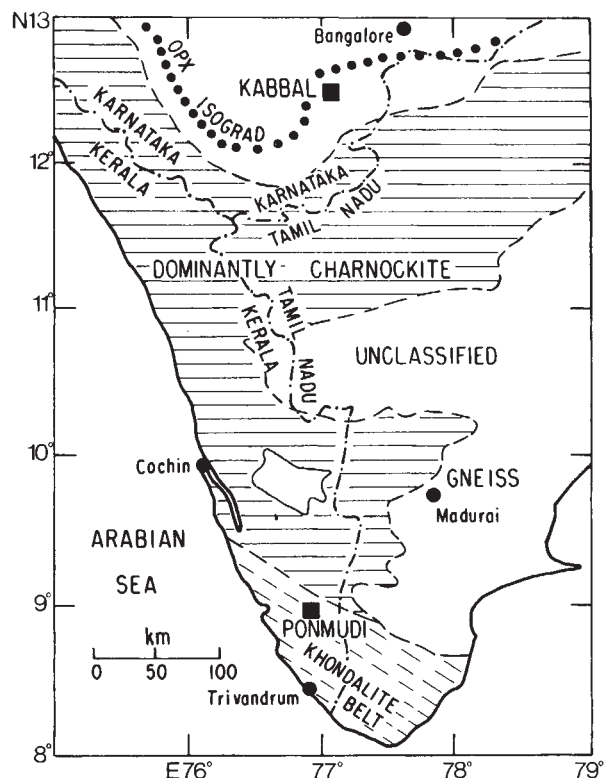


Fig. 1 Locations of Ponmudi, Kerala, and the Precambrian geology of southern India. Bangalore is near the dividing line between terrains of amphibolite facies and granulite facies.

the pressures and perhaps the temperatures are higher than the 750 °C and 5.4 kbar inferred for the first appearance of orthopyroxene at Kabbaldurga^{15,16}.

Quartz crystals in the Ponmudi charnockites contain many arrays of CO_2 -rich fluid inclusions; they contain also a significant proportion of decrepitated and partially-decrepitated inclusions. CO_2 -rich fluid inclusions are also found in non-charnockitic gneisses, although not as abundantly as in the charnockites. A reconnaissance survey of the charnockite fluid inclusions showed freezing points 1–2 °C below the triple point (-56.6 °C) of pure CO_2 . This must result from the presence of another component miscible with CO_2 , most probably CH_4 or CO because of the abundance of graphite. If the dilutant is CH_4 , the amount could be as much as 16 mol (ref. 17). Homogeniz-

Table 1 Whole-rock and mineral analyses of adjacent pairs of grey gneisses and charnockites from Ponmudi

wt %	18-6 w-r gneiss	18-6 w-r charn.	18-8 w-r gneiss	18-8 w-r charn.	18-6 garnet gneiss	18-6 garnet charn.	18-6 biotite gneiss	18-6 biotite charn.	18-6 opx charn.
SiO_2	65.63	67.06	64.82	65.07	36.8	36.9	35.7	35.5	48.9
TiO_2	0.97	0.97	0.78	0.85			5.3	5.2	
Al_2O_3	15.18	15.20	15.88	14.83	21.2	21.4	13.6	13.8	1.8
FeO	6.87	5.29	6.24	6.30	34.6	34.4	20.1	20.3	38.6
MnO	0.10	0.06	0.08	0.06	0.5	0.6			0.1
MgO	1.13	0.95	1.11	1.18	4.3	4.3	10.3	10.5	11.3
CaO	2.24	2.40	2.10	2.07	2.4	2.4			0.1
Na_2O	2.33	2.70	2.31	2.46					
K_2O	4.03	4.60	5.06	5.42			9.8	9.0	
P_2O_5	0.20	0.20	0.17	0.20					
LOI	1.07	1.17	0.87	0.81					
Total	99.75	100.6	99.42	99.25	97.8	100.0	94.0	94.3	100.8
p.p.m.									
Rb	207	186	247	228			Mg/(Mg + Fe)		
Sr	142	165	166	184	0.181	0.184	0.477	0.480	0.343
Y	60	47	80	54					

Composition of plagioclase: in gneiss, 32.0 mol % anorthite (An); in charnockite, 32.5 mol % An. Also present, alkali-feldspar, quartz, graphite, ilmenite, apatite. w-r, Whole rock; opx, orthopyroxene; LOI, loss on ignition.

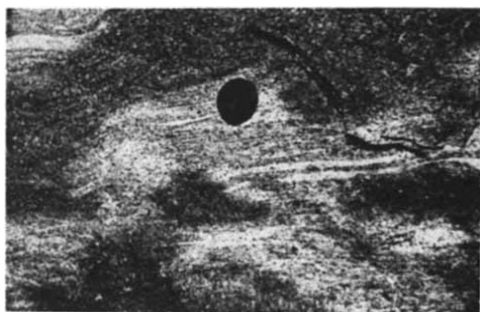


Fig. 2 Charnockite alteration (dark) of garnet-biotite gneiss (lighter), showing crosscutting and disruption of gneissic foliation.

ation temperatures ranged from +10 to +17 °C with the largest peak at +11 °C. This corresponds to pressures between 3.3 and 4.0 kbar for a temperature of 760 °C¹⁸ (a maximum estimate because of the miscible component in the CO₂). The low pressure may indicate entrapment at high temperature during uplift following peak metamorphism. Near-isothermal uplift could cause decrepitation if pressures fall 1–2 kbar below the entrapment isochores¹⁹.

The reaction producing orthopyroxene at Ponmudi is essentially biotite + quartz → orthopyroxene + K-feldspar + H₂O; garnet does not appear to participate. This reaction is at least divariant because of the complex vapour phase and the presence of Ti in the biotite. Orthopyroxene forms from amphibole breakdown at Kabbaldurga, where biotite is stable with quartz. Hence we infer the formation at Ponmudi is of higher grade than at Kabbaldurga, which is supported by the occurrence of inter-layered orthocharnockites in the khondalite belt of Kerala and Tamil Nadu²⁰; bulk chemistries that could yield amphibole-bearing acid gneisses at lower grade exhibit orthopyroxene in the khondalite belt.

Progressive metamorphism is usually explained as a response to increases in temperature and/or pressure. However, it is unlikely that large differences in the metamorphic pressures and temperatures occurred over the short distances between the biotite-bearing and orthopyroxene-bearing patches at Ponmudi. In one popular model of high-grade metamorphism, granulite facies mineral assemblages are produced as the residues of partial melting²¹. The isochemical nature of the metamorphism precludes this for Ponmudi. The mineral assemblage in the charnockites requires low water pressures, probably of ~10–20% of the total pressure¹⁷, which may be fundamental in understanding the development of charnockite at Ponmudi. An influx of water-deficient fluid during the high-grade metamorphism would lower water fugacities, promoting the breakdown of biotite and the formation of orthopyroxene. Such fluids would probably be channelled in zones of enhanced permeability, such as in shears, which could explain the localized development of charnockite. The CO₂ densities in fluid inclusions at Ponmudi are too low to be consistent with peak metamorphic pressures and may have been affected by deformation during postmetamorphic uplift.

A mixed charnockite-grey gneiss quarry near Panaikkudi, Tamil Nadu has been recently reported²² and although few petrologic details were given, the occurrence seems similar to that at Ponmudi. We note small amounts of charnockite cutting biotite-garnet paragneiss in a few other widely-separated localities in Tamil Nadu and Sri Lanka, but most of these are complicated by some late retrogression and hence are not as straightforward as at Ponmudi. However, it appears that arrested charnockitization of biotite-garnet paragneiss may be widespread over the high-grade terrain of southern India that is exposed for thousands of square kilometres, indicating that metamorphic conditions throughout most of this area remained fairly close to those required for the first appearance of orthopyroxene in these biotite-garnet paragneisses ($T \sim 760$ °C, $P \sim 6.0$ kbar, $P_{H_2O} < 0.2 P_{total}$). Relatively-small local fluctu-

ations in these conditions would explain the relative distribution of biotite-garnet paragneisses and their orthopyroxene-bearing equivalents. Widespread occurrence of arrested charnockite also indicates that carbonic metamorphism occurred throughout this crustal level. However, the mechanism of transport of large quantities of CO₂ into such a geographically-broad metamorphic environment remains an unresolved problem.

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Extreme isotopic homogeneity among basalts from the southern East Pacific Rise: mantle or mixing effect?

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Basaltic lavas which have erupted along the great oceanic ridge system are characterized by their overall chemical and isotopic homogeneity. Nevertheless, they show evidence for variability in the chemical and isotopic composition of the ocean ridge basalt (ORB) source whose scale and distribution remain uncertain. Isotopic compositions of Sr, Nd, Pb and Hf and ratios of some highly incompatible elements have been used as tracers of ORB source compositions, usually permitting source characteristics to be distinguished from petrogenetic effects. The Mid-Atlantic Ridge (MAR), in particular, shows correlations in the isotopic compositions of several elements, between isotopic compositions and topography and between these parameters and incompatible element concentrations and ratios^{1–3}. We report here isotopic measurements for basalts, dredged between approximately 30 and 34° S on the East Pacific Rise and adjacent Maddingly Rise, which show highly uniform Sr and Nd isotopic compositions, possibly resulting, in part, from mixing associated with a high rate of magma generation and rapid spreading (~170 mm yr⁻¹). However, a ridge segment of comparable length between 18 and 22° S and with only a slightly lower spreading rate shows much greater isotopic variability. Hence we are apparently sampling small but detectable regional differences in mantle composition. The isotopic homogeneity among the basalts from 30 to 34° S implies that any heterogeneity in the underlying upper mantle is uniformly distributed on a scale much smaller than the volume sampled in individual magma batches, and is thus averaged out in the resulting lavas.

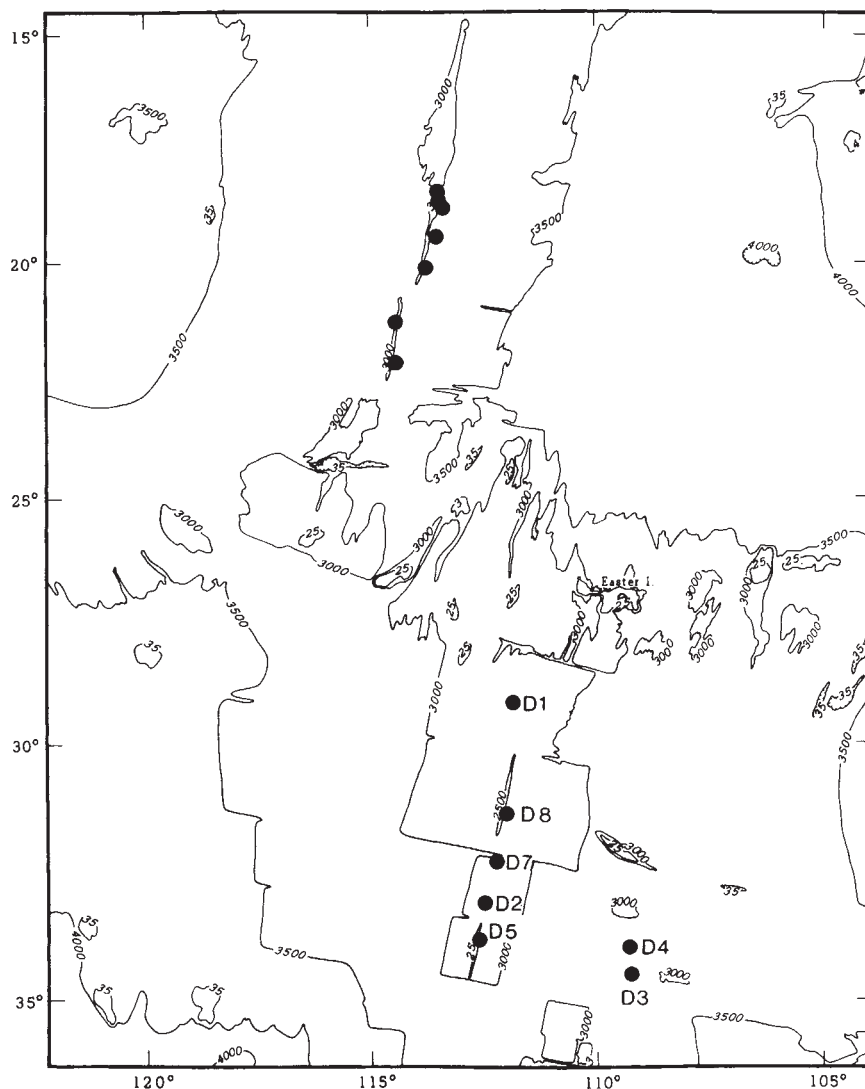


Fig. 1 Bathymetric map of the EPR between 15° and 35° S showing the locations of Pascua expedition dredge samples measured here. Dredges 3 and 4 are on Maddingly Rise¹². Contouring is in metres. Solid circles without dredge numbers indicate locations of dredges between 18° S and 22° S discussed in the text. Bathymetry is after Mammerickx and Smith¹³.

Large-scale regional heterogeneities, for example, near Iceland or in the Azores, have been ascribed to plumes or blobs of relatively-enriched material rising from great depths in the mantle^{3,4}. Individual ORB analyses, however, occasionally show isotopic compositions as extreme in $^{87}\text{Sr}/^{86}\text{Sr}$ or $^{143}\text{Nd}/^{144}\text{Nd}$ as these, despite their origin from areas lacking topographic or other manifestations of plume activity⁵. It has been suggested that the much greater isotopic variability observed among MAR basalts compared with samples from the East Pacific Rise (EPR) is a function of less efficient mixing at slow-spreading ridges (MAR) than at fast-spreading ridges (EPR)^{1,6-8}. If true, this has important implications for the dimensions of small-scale isotopic

heterogeneities in the ORB source, as the spreading-rates between areas of high and low-Sr isotopic variability differ by only a factor of two or three⁸. Mixing volumes must differ by similar factors unless melt generation and storage are quite different in different spreading-rate regimes (for which there is no firm evidence). Thus, if this hypothesis is correct, the size of the mixing heterogeneities is such that a moderate difference in spreading rate (for example, from $\sim 2 \text{ cm yr}^{-1}$ for parts of the North Atlantic to $\sim 6 \text{ cm yr}^{-1}$ for the northern EPR) will result in a considerable change ($\sim 6\times$)⁸ in Sr isotopic variability in erupted basalts.

Our data for the basalts dredged between 30 and 34° S are

Table 1 Isotopic data for Pascua dredge samples

	Sr	Rb	$^{87}\text{Sr}/^{86}\text{Sr}$	Nd	Sm	$^{143}\text{Nd}/^{144}\text{Nd}$	ϵ_{JUV}
PA3-D1-F	101	0.51	0.702519 ± 26	9.62	3.50	0.513087 ± 18	10.2
PA3-D2-B	122	0.83	0.702492 ± 26	15.9	5.18	0.513089 ± 14	10.2
PA3-D3-D	122	1.01	0.702500 ± 26	12.9	4.41	0.513090 ± 14	10.2
PA3-D4-A	117	0.99	0.702528 ± 26	11.6	3.95	0.513091 ± 14	10.2
PA3-D5-G	121	0.24	0.702456 ± 26	8.07	2.90	0.513094 ± 14	10.3
PA3-D7-A	109	0.39	0.702474 ± 26	7.15	2.54	0.513089 ± 16	10.2
PA3-D8-D	116	1.20	0.702500 ± 26	17.8	6.05	0.513093 ± 16	10.3

The analyses of all samples, from dredge collections made during Pascua expedition of the Scripps Institution of Oceanography, were carried out on rigorously-cleaned fresh hand-picked glass fragments leached briefly in 2 N HCl to ensure that no seawater components remained on the surfaces. All concentrations are in p.p.m. Isotopic data are fractionation-corrected to $^{87}\text{Sr}/^{86}\text{Sr} = 0.1194$ and $^{148}\text{NdO}/^{144}\text{NdO} = 0.242436$. Measured standard values are: $^{87}\text{Sr}/^{86}\text{Sr} = 0.71026$ for NBS 987 Sr, with a total range of ± 0.000026 , and $^{143}\text{Nd}/^{144}\text{Nd} = 0.511859$ for the La Jolla Nd standard, with a total range of ± 0.000014 . Where statistical errors for an individual sample run were less than the measured range for our isotopic standard, the error given corresponds to the observed standard range. A value of $^{143}\text{Nd}/^{144}\text{Nd} = 0.512566$ corresponds to $\epsilon_{\text{JUV}} = 0$.

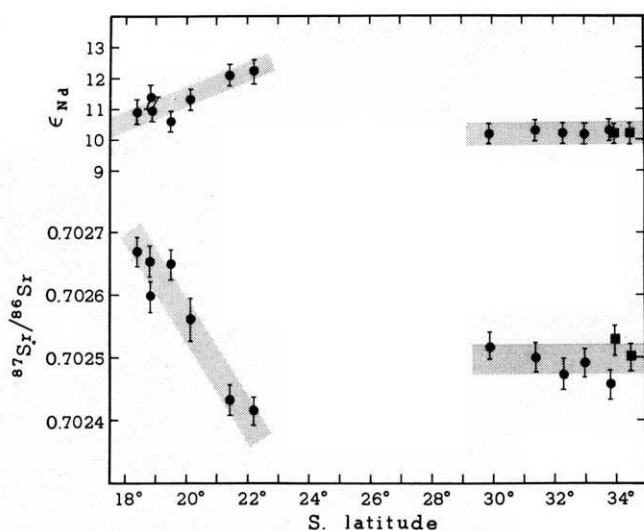


Fig. 2 Isotopic data for the Pascua expedition dredge samples plotted as a function of latitude. Also shown for comparison are our unpublished data for a ridge segment of comparable length north of Easter Island. Note the striking uniformity of the Pascua data, particularly for Nd (expressed in ϵ -units). Details of the isotopic measurements are given in Table 1.

given in Table 1 and dredge locations are shown in Fig. 1. The southern EPR has a complex spreading history^{9,10} with spreading rates among the highest observed anywhere, $\sim 170 \text{ mm yr}^{-1}$ (ref. 11). Our samples—all fresh glassy basalts from present-day spreading centres—were collected along $\sim 550 \text{ km}$ of the EPR near its intersection with the Chile Rise. Two of them (PA3-D3D and PA3-D4A) are from the Maddingly Rise which forms the eastern boundary of the Juan Fernandez microplate¹², and is believed to be an active ridge segment (although the spreading rate is low) based on earthquake epicentres¹⁰, the freshness of the collected basalts and sea-floor magnetics (H. Craig, personal communication). North of this region only the EPR has been active during the past 9 Myr and fossil portions of an older continuation of the Chile Rise occur east of the EPR to a latitude of about 10° S ¹⁰.

The isotopic data are shown as a function of latitude in Fig. 2 together with our unpublished data for basalts from the EPR north of Easter Island. The most striking feature of the Pascua (southern) results is their very small range of variability for both Sr and Nd isotopes: the total range of Nd isotopic composition for the seven samples is 0.000007, or 14 p.p.m., only one quarter that of our La Jolla Nd standard (Table 1); the Sr isotopic variability is larger and is slightly greater than for our Sr standard. This may indicate some small but real variability in the samples but the range is still very small—less than half that observed among a group of ridge basalts collected along a small

segment ($\sim 6 \text{ km}$) of the EPR at 21° N and much less than that for basalts from the ridge segment of comparable length north of Easter Island (Fig. 2).

Our data suggest that the upper mantle supplying magmas to this segment of the EPR is isotopically homogeneous on a scale ranging from less than the volume affected by an individual melting event to that of the entire sample region. That these samples represent a series of separate magma batches is indicated not only by their wide geographic distribution and location on discrete ridge segments separated by transform faults (Fig. 1), but also by the variations in their major element compositions (Table 2): two (D2-B and D8-D) are highly fractionated Fe-Ti-rich lavas, which is also reflected in their high Sm and Nd contents (Table 1). However, the variation in Sm/Nd among all samples is random and only $\sim 10\%$.

Because of the very high spreading rate, it is tempting to attribute the extreme isotopic uniformity to mixing associated with a high rate of magma production—indeed this may partly account for the observations. However, the spreading rate at $18\text{--}22^\circ \text{ S}$, where samples from a similar length of ridge show much greater isotopic variability (Fig. 2), is only marginally lower than at $30\text{--}34^\circ \text{ S}$ (ref. 11) and, although only two of our samples come from the slower-spreading Maddingly Rise¹², their isotopic compositions are identical to those from the adjacent EPR. Thus, whereas mixing at fast-spreading ridges (EPR) may homogenize isotope distribution in magmas on the scale of individual melt batches and so obscure small-scale mantle heterogeneities, it cannot remove regional heterogeneities. This is consistent with the data from the $18\text{--}22^\circ \text{ S}$ segment which exhibit a regular trend in isotopic composition (Fig. 2). The data for this region suggest that homogenization occurs only on a scale $\leq 0.5\text{--}0.75^\circ$ latitude ($\sim 55\text{--}85 \text{ km}$) which, if corroborated by more detailed sampling, would constrain the size of individual melt batches.

The isotopic homogeneity of the $30\text{--}34^\circ \text{ S}$ samples is exceptional. Its areal extent cannot be estimated precisely as all the samples are from the narrow presently-active spreading centre, but, because those from the Maddingly Rise and the EPR are isotopically indistinguishable, considerable lateral extent is indicated. A reasonable areal estimate is $2 \times 10^5 \text{ km}^2$; if a conservative 2-km thick section was generated by 15–20% partial melting, the volume of isotopically-uniform mantle would be at least $2.5 \times 10^6 \text{ km}^3$. The average Nd isotopic composition is slightly lower than those we have measured for almost any other portion of the EPR between 21° N and 23° S , with the exception of some transitional and alkali-enriched basalts from the Siqueiros Fracture Zone (our unpublished results); Sr isotopic ratios are less distinct. Thus, our data indicate that there is a large mantle region near the intersection of the EPR and the Chile Rise which is isotopically uniform to a scale much less than those of individual melt batches, but which nevertheless is isotopically distinct from other sections of the EPR and that such subtle variations in average isotopic composition cannot entirely result from differences in the spreading rate.

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Table 2 Chemical composition of samples

	D1-F	D2-B	D3-D	D4-A	D5-G	D7-A	D8-D
SiO ₂	50.8	49.7	49.9	49.2	49.9	49.5	50.3
TiO ₂	1.61	2.28	1.97	1.73	1.42	1.18	2.66
Al ₂ O ₃	14.4	13.8	14.5	15.8	15.3	15.2	13.2
FeO	10.7	12.4	11.1	10.3	10.1	9.25	14.1
MgO	7.12	6.45	6.79	8.23	7.76	8.04	5.62
MnO	0.24	0.21	0.19	0.19	0.21	0.18	0.23
CaO	11.7	11.2	11.2	11.4	12.5	12.5	10.2
Na ₂ O	2.60	2.84	2.84	2.49	2.68	2.34	2.52
P ₂ O ₅	0.12	0.19	0.20	0.16	0.11	0.08	0.20
K ₂ O	0.07	0.13	0.10	0.11	0.07	0.07	0.15
Sum	99.36	99.20	98.79	99.61	100.05	98.34	99.18

Microprobe analyses of glass using Smithsonian mineral standards. The data are normalized to Juan de Fuca basalt glass VG-2.

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Hydrothermal vents on an axis seamount of the Juan de Fuca ridge

Canadian American Seamount Expedition*

The Juan de Fuca Ridge, a 500-km section of the mid-ocean ridge system, is bounded by the Sovanco and Blanco fracture zones^{1,2}. Although the ridge spreads at a medium rate (29 mm yr⁻¹ half-rate)³, a significant portion of its crest has a morphology typical of faster spreading ridges; the axial valley is 1–2 km wide within a central horst structure⁴. The ridge crest shoals to 1,500 m at the intersection with the Cobb–Eickelberg Seamount chain forming a broad edifice called 'Axial Seamount' (Fig. 1). Previous studies both north and south of Axial Seamount demonstrated hydrothermal activity associated with the shallowest portions of each of the ridge segments along the Juan de Fuca^{5–9}, a similar coincidence to that noted for the East Pacific Rise¹⁰. We report here the results of the first manned submersible expedition to the Juan de Fuca Ridge, which found hydrothermal activity and at least 14 new vent animals in the caldera of Axial Seamount.

During this joint Canada–US expedition, the Canadian submersible Pisces IV completed eight dives with equipment including an externally-mounted stereo camera, a high-temperature probe, titanium water-sampling bottles, devices for sample collection and an observer-held video camera.

The summit of Axial Seamount is a flat-floored caldera, breached at its southern end (Fig. 1). Seamarc I images reveal fissuring and faulting at the intersection of the northern caldera wall and the spreading axis¹¹, the focus of our study, where a thermal anomaly has been measured¹². North of the study site, there were fissures towards the south 2–10 m wide and 1–20 m deep, occupying a zone 400 m wide; pelagic sediments were up to 20 cm thick. The exposed and truncated pillow basalt of the caldera wall seemed older than that of the floor. Steep talus slopes along its base were overlapped occasionally by sheet flows of the floor. The northwestern caldera floor was lightly-sedimented glassy basalt with areas of older pillow basalt. Drain-back features with relief to 5 m were common and included collapsed roofs, hollow basalt columns and cavitied and buckled sheet flows.

The fissures on the caldera wall were replaced by one major fissure on the floor (Fig. 2) of ~300-m length, terminated in the north by a talus pile and in the south by a vertical wall beyond which no traces of rifting were found. Only the northern 200 m of the fissure were hydrothermally active with flows greatest at the vents indicated in Fig. 2 where fluids up to 35 °C were discharging through mounds of pogonophoran worms. Lower temperatures (4–7 °C) were measured in cavities of diameter 1 m, where hydrothermal precipitates were essentially absent and the fissure basalts showed no obvious alteration.

East of the fissure (Fig. 2), three chimneys (sulphides and sulphates) rose to 12 m; one pagoda-shaped chimney was still venting water (measured to 19 °C) from lateral flanges and an adjacent spire, 1.7 m high and weighing 160 kg, was collected. The pillars were aligned N–S, but no tectonic features connecting them with an area of diffuse venting 50 m south were found. The onlapping nature of the glassy sheet flows at the chimney bases and the lack of sulphide debris suggest that the lava flows were recent with the major vent fissure being a channel through which the lava may have drained.

Sulphates and sulphides around worm tubes give the chimney samples a spongy texture. The tubes are partially filled by

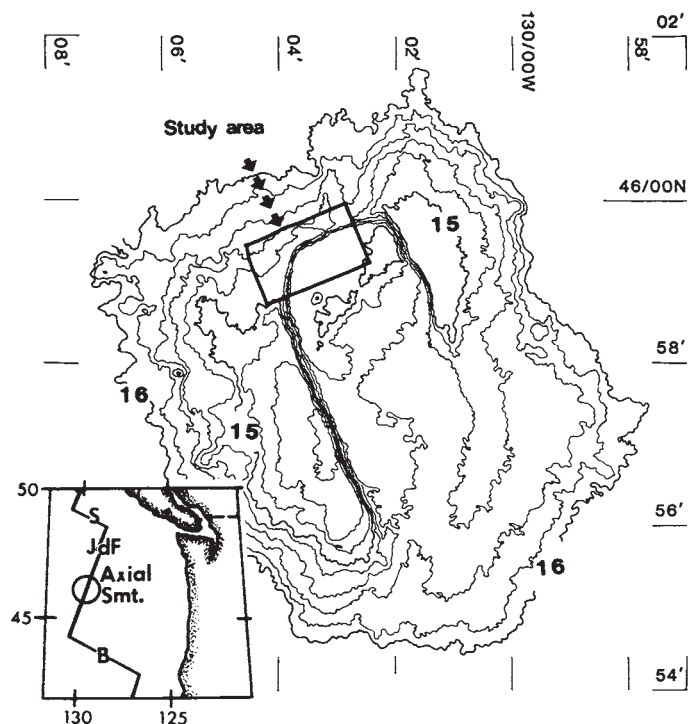


Fig. 1 NOS Seabeam bathymetry of Axial Seamount and Pisces IV study area. Contour interval is 20 m with labels in 100 m. Seamount rises to 1,500 m are compared with flanking ridge axes at 2,200–2,700 m. Caldera measuring about 7 km × 3 km with a flat floor at 1,570 m had a long axis along 340°, oblique to the 020° strike of the Juan de Fuca Ridge. The inset illustrates the setting of Axial Seamount with respect to the Juan de Fuca Ridge and the North American coast. Note the general ridge trend. S = Sovanco fracture zone; JdF = Juan de Fuca ridge; B = Blanco fracture zone.

amorphous silica, with barite constituting 80% of the total and very low anhydrite, suggesting redissolution (Table 1; ref. 13). Zinc sulphide contains galena and intermediate solid solution; textures and galena dendrites indicate rapid crystal growth. This mineralogy must have been produced by temperatures >150 °C¹⁴. The chemical analyses (Table 1) are similar to others reported from this ridge^{9,7} with samples tending to be rich in zinc and silver and poor in copper.

Fluid from vent A has an origin similar to that of the Galapagos springs where deep primary hydrothermal fluid ascends and mixes below the sea floor with local seawater¹⁵ whose proportion may be increased on sampling; assuming only two end members and no magnesium in the primary fluid¹⁵, the magnesium content (Table 2) suggests the sample is 5% primary fluid and 95% seawater. Temperature relationships remain uncertain; extrapolation of the 29 °C vent temperature gives 532 °C for the primary fluid, with quartz thermometry giving a better estimate¹⁵. The [Si], extrapolated to [Mg]=0, is 18.6 mmol kg⁻¹, consistent with a temperature of 300–350 °C.

In most respects the chemistry is similar to that of other vents^{15–18}, but the helium-3 enrichment is markedly different. Global chemical fluxes can be estimated from the element/He systematics of individual vents¹⁷. Vent A fluid has a ³He/⁴He ratio of (8.11 ± 0.15) times atmospheric value, similar to local basaltic-glass values (8.22, 8.36). The absolute ³He enrichment calculated from the measured ³He/Ne ratio (assuming Ne behaves conservatively) is very high: 580 times the value for air-saturated seawater, in contrast with enrichments of 110 times at Galapagos¹⁷ and 135 times at 21° N (ref. 18) normalized to

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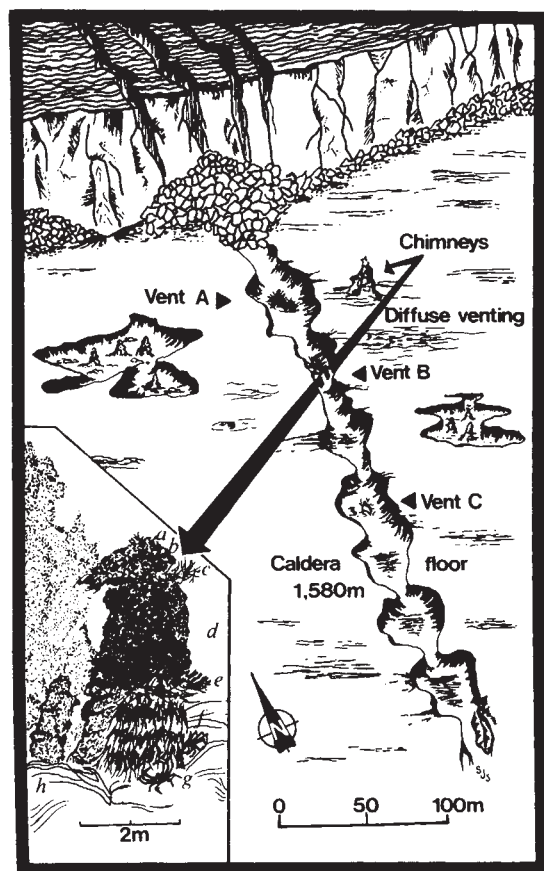


Fig. 2 Drawing of the northern caldera wall and floor on Axial Seamount. Extensive submersible searches revealed fissuring only in a limited zone on the caldera wall. On the floor, the single fissure, trending N-S, comprised a series of depressions 15–25 m wide, 30–50 m long and 5–20 m deep. Concentrated hydrothermal activity was present at three 'vents'. Hydrothermal flows were found east of the fissure on a sulphide chimney and in an area of cracked basalts. Inset: The active sulphide chimney was densely populated in distinct zonation with respect to the warm water flow. *a*, Protozoan colonies (folliculinid ciliate); *b*, polynoid polychaetes; *c*, pogonophorans (white tubes); *d*, limpets stacked one above the other in numbers estimated up to 10^5 on this structure; *e*, pogonophorans and alvinellid polychaetes; *f*, small gold pogonophorans; *g*, majid crabs observed feeding on the pogonophorans. Water flowed from under the ledges at *c* and *e*. The small spire *h* was recovered intact.

the same magnesium concentration. Evidently, ^3He -heat ratios may show sufficient variation to make global flux estimates very uncertain.

The Axial Seamount fluid has higher alkalinity than local seawater, its relatively acid pH (6.18 versus 6.65 in an equivalent Galapagos fluid), at which iron remains in solution despite the high sulphide concentration, results from its high inorganic carbon content. The CO_2 enrichment (40 mmol kg^{-1}) is similar to that of helium and the source may be magmatic. Alkalinity may have been added during subsurface mixing¹⁹; in laboratory seawater-basalt experiments at 300°C , alkalinities of up to 1.3 meq kg^{-1} have been generated.

Dense thickets ($4\text{--}6 \text{ m}^2$) of pogonophorans overgrew major vents in the fissure, whereas minor vents were surrounded by bacterial mats, small prone pogonophorans and limpets. Colonial protozoans and bacterial mats colonized most surfaces of the active fissure sections and localized bacterial mats delineated areas of diffuse venting south of the sulphide chimneys. The density of vent animals on the one active chimney was high (Fig. 2).

Table 1 Composition of chimney rock samples

Minerals in decreasing order of abundance*		
Major	Minor	Trace
Barite	Wurtzite	Galena
Amorphous silica	Pyrite	Cu-Fe ISS†
Sphalerite†	Anhydrite	Tetrahedrite
Marcasite		
Chemical analysis§		
Weight %	Sample 1	Sample 2
Zn	10.1	28.2
Fe	5.8	3.2
Cu	0.1	0.3
Pb	0.6	0.1
Ba	15.0	14.2
Ca	0.2	0.1
SiO_2	41.4	20.4
CO_2	0.3	0.2
p.p.m.		
Ag	342	233
Cd	110	740
Mo	45	32
Co	3	3
Ni	32	11
Mn	470	1,300

In addition to the small spire, three hand specimens were also retrieved.

* Minerals identified by transmitted and reflected light microscopy and X-ray diffraction. († Three electron microprobe analyses measured elemental quantities by weight: Zn, 61.3%; Fe, 4.6%; Cu, 0.7%; S, 33.4%. ‡ Five samples of intermediate solid solution were analysed: Zn, 1.6%; Fe, 29.1%; Cu, 33.5%; S, 35.8%.)

§ Bulk chemical analysis. (Methods of analysis: Zn, Fe, Ba, Ca, SiO_2 by X-ray fluorescence; Cu, Pb, Ag, Cd, Mo, Co, Ni, Mn by direct-coupled plasma; CO_2 by wet chemistry. Sample 1, unoxidized; sample 2, oxidized.)

Table 2 Fluid chemistry

Component	Vent A Axial Seamount	Local seawater (1,600 m)
Mg (mmol kg^{-1})	49.39	52.04
$(^3\text{He}/\text{Ne})/(^3\text{He}/\text{Ne})_{\text{std}}$	580	1.5
Si (mmol kg^{-1})	1.10	0.16
Cl (mmol kg^{-1})	531.9	538.6
Ca (mmol kg^{-1})	11.06	10.13
SO_4 (mmol kg^{-1})	25.94	27.83
pH	6.18	7.7
Alk (meq kg^{-1})	2.66	2.42
ΣC (mmol kg^{-1})	4.32	2.37
Fe ($\mu\text{mol kg}^{-1}$)	2.6	—
H_2S ($\mu\text{mol kg}^{-1}$)	330	—
Mn ($\mu\text{mol kg}^{-1}$)	27.7	—
Li ($\mu\text{mol kg}^{-1}$)	58.0	28
Rb ($\mu\text{mol kg}^{-1}$)	4.4	1.3
Ba ($\mu\text{mol kg}^{-1}$)	1.31	0.15
Sr ($\mu\text{mol kg}^{-1}$)	92 ± 3	88

Fourteen new vent metazoans were recovered in three samples. The pogonophoran (length $\leq 1.5 \text{ m}$, diameter $\leq 2 \text{ cm}$) is a new vestimentiferan taxon (M. L. Jones, personal communication). Ultrastructural analyses have confirmed that the bacteria in the trophosomal tissues are definitely intracellular²⁰. Two alvinellid polychaetes are new species in the genus *Paralvinella*²¹; the only animal described from tropical vents is an ampharetid polychaete species, *Amphisamytha galapagensis*²². Other new species include two polynoid polychaetes (D. Weston, personal communication), three gastropods in new subfamilies under description (J. McLean, personal communication) and a copepod from the pogonophoran tubes²³; a few tiny bivalves seem to be related to clam and mussel families

from other vents (F. Bernard, personal communication); a vent-associated fish was not collected and galatheid and majid crabs, common on the periphery of the vents, are known deep-water species. More extensive descriptions of the Axial Seamount organisms can be found elsewhere²⁰.

Large quantities of mucus bound the pogonophoran tubes together and provided habitat for many small organisms. The mucus, containing predominantly bacterial filaments but also coccoid bacteria (0.5–1.0 μm in diameter), fragmented alvinellid tubes, diatom frustules and basalt fragments, was intimately associated with alvinellid polychaetes and may be produced by them. The mucus was incubated on board ship in filtered vent water with $\text{NaH}^{14}\text{CO}_3$ to detect dark- CO_2 fixation. The measured rate of CO_2 incorporation ($86.3 \text{ mg-C kg-dry wt}^{-1} \text{ day}^{-1}$; $n=6$, s.d. = 23.4) suggests that these bacteria are significant primary producers. The rate of CO_2 fixation by bacteria in the same emitted vent water collected for chemical analyses gave a value of $2.42 \text{ mg-C m}^{-3} \text{ day}^{-1}$, which is in the range measured in waters of the Galapagos vents²⁴. Presence of thiosulphate at 1 mM

increased CO_2 incorporation to $3.7 \text{ mg-C m}^{-3} \text{ day}^{-1}$ and, at 10 mM, the rate was $4.4 \text{ mg-C m}^{-3} \text{ day}^{-1}$. This response indicates that sulphide-oxidizing bacteria were active in the vent water. Bacterial cell numbers, counted by epifluorescent microscopy, were $3.5 \times 10^4 \text{ cells cm}^{-3}$, significantly lower than those reported for Galapagos vent waters^{24,25}.

The unique features of Juan de Fuca hydrothermal systems will be investigated further with data from the expedition to the ridge in the summer of 1984.

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A groundwater source of nitrate in nearshore marine sediments

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Contamination of groundwater is a pervasive and serious problem in many developed coastal areas^{1–3}, but potential interactions of groundwaters with, and their impact on, adjoining coastal waters have generally not been considered. Although submarine groundwater discharge could be an important source of nitrogen to coastal marine environments⁴, there has been no direct evidence for this. Recently, subsurface discharge has been shown to account for about 10–20% of the freshwater input to Great South Bay, New York⁵. The upper aquifer is the presumed source of this discharge and, because it is heavily contaminated with nitrate⁶, we suggest that groundwater is an important source of nitrate to the bay. Our determinations of the interstitial nutrient chemistry of freshly collected cores from nearshore sediments support this hypothesis. Although highly variable in time, cores taken after substantial rainfall showed increasing nitrate concentrations and decreasing salinities with depth. We conclude that submarine groundwater discharge is indeed a source of nitrate which should be considered in estimates of nitrogen influx to coastal marine ecosystems.

Pore-water nutrient profiles were determined in Great South Bay, New York, at a site off Roe Avenue, near Patchogue, Long Island. The water depth was ~1 m at this site, which was sandy with sparse eelgrass beds; high rates of submarine groundwater discharge, typically $10\text{--}50 \text{ l m}^{-2} \text{ day}^{-1}$, have been recorded there and elsewhere in nearshore sediments of Great South Bay^{5,7}.

Core samples taken on 3 December 1982 showed slight decreases in salinity and concomitant increases in nitrate below 20 cm (Fig. 1a), but those taken on 16 December 1982 (Fig. 1b)

had a more typical profile for a marine sediment: salinity was uniform with depth, whereas ammonium increased and nitrate decreased. However, on 18 and 20 April and 10 May 1983 (Fig. 1c, d and e), there were substantial decreases in salinity with depth and, for marine sediments, very high concentrations of nitrate through the whole sediment column indicating, together with the submarine groundwater discharge data^{5,7}, a highly dynamic and variable situation. An exponential decrease in groundwater discharge rates with increasing distance from the shore has been reported^{5,7}. We therefore took several offshore transects of interstitial nitrate after periods of substantial rainfall (Fig. 2); lowest salinities and highest nitrates occurred in samples closest to shore.

An anoxic zone with sulphate reduction between 5 and 10 cm was always noted in our core samples (unpublished data) and active nitrification above it⁸, possibly accounting for some of the anomalies in the profiles of nitrate and ammonium above 5 cm (for example, the nitrate minimum and sharp increase near the surface, Fig. 1c). For each of the samples, mean nitrate and salinity were therefore compared for depths below 15 cm and were well correlated (Fig. 3). The median concentration of nitrate in the upper glacial aquifer in Suffolk County between 1972 and 1976 was $\sim 100 \mu\text{M}$ ⁵ which agrees well with the estimate of $66 \mu\text{M}$ at zero salinity calculated from the regression line of Fig. 3.

The rate of submarine groundwater discharge is a function of the hydraulic gradient^{3,4} maintained by rainfall and heavy rain causes rapid rate increases⁷. Mean nitrate and salinity (15–40 cm) were plotted also against the rainfall preceding the date of sampling. Although there was no obvious correlation with precipitation over the 3 or 10-day periods before sampling, good correlations were obtained between interstitial nitrate or salinity and rainfall when considering the 30-day integrated rainfall (Fig. 4), a further indication that nitrate is indeed imported by the groundwater.

Combined nitrogen, which regulates algal growth and productivity in many marine environments^{9,10}, enters coastal waters

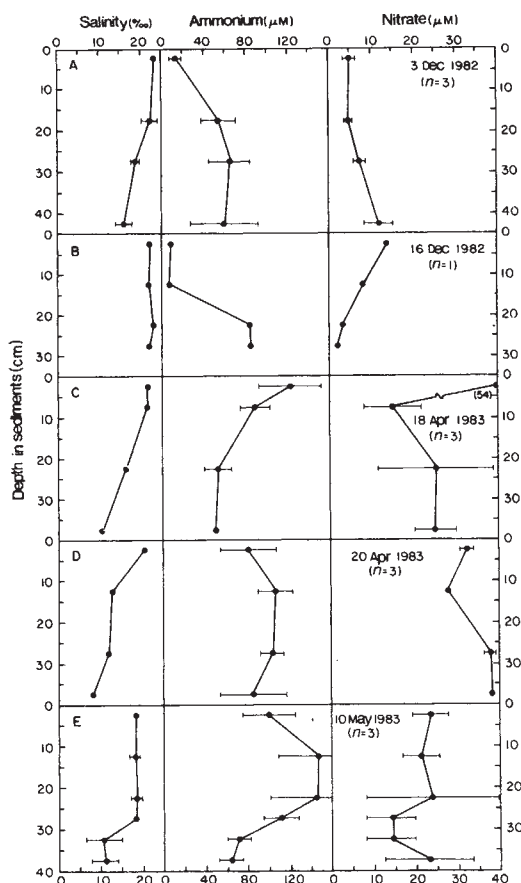


Fig. 1 Distributions of salinity, ammonium and nitrate in sediments of the site off Roe Avenue. All samples were collected using clear plastic core tubes of 5.6 cm internal diameter. In the laboratory, four to six 5-cm sections were taken from several horizons of each core and pore waters were collected by centrifuging through Delrin pore water separating bottles (B. Brinkhuis, patent pending) at 2,500 r.p.m. for 30 min. Salinity profiles were determined from the pore waters of cores with a Goldberg refractometer. Nutrients (NO_3^- , NO_2^- , and NH_4^+) were analysed by standard methods³¹ adapted for the Technicon Autoanalyzer³². Results, except those for 16 December, are the means from 3 cores $\pm$ standard error.

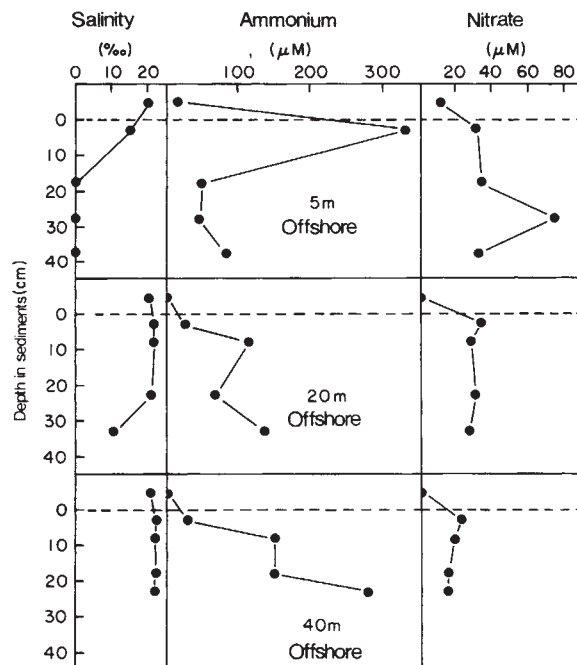


Fig. 2 Interstitial salinity, ammonium and nitrate of a transect at Roe Avenue site taken on 22 September 1983. Procedures, as Fig. 1.

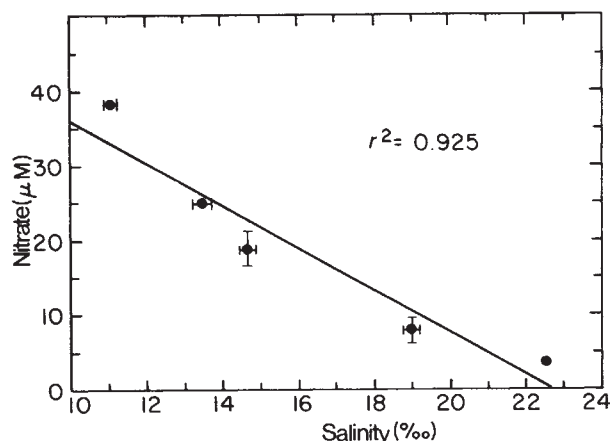


Fig. 3 Correlation of interstitial nitrate with salinity. Values of nitrate and salinity are means in the range 15–40 cm for five core samples from Fig. 1.

through several recognized routes, including riverine flow, runoff and industrial and domestic sewage discharge, all obvious and measured sources of particulate and dissolved nitrogen^{10,11}. However, groundwater importation has not been evaluated in most studies aimed at budgeting nitrogen inputs to coastal waters, probably because the areal extent and volume discharge of groundwater in coastal areas is generally small compared with other fresh water inputs¹². Also, in the past, this form of transport of nitrogenous solutes was probably minor as the extreme concentrations reached in some aquifers is only a recent phenomenon^{1,2}, forcing us to reconsider the potential importance of submarine groundwater discharge of nitrate associated with population growth, urbanization and increased use of coastal areas.

Submarine groundwater seepage should occur in any coastal area with an adjacent landward aquifer and has been seen along the continental slope of the eastern United States¹³ and the coasts of Florida¹⁴, North Carolina¹⁵, Long Island^{5,7} and Western Australia⁴. The general importance of groundwater importation of nitrogen in any location depends on rainfall, soil permeability, water-table height and contaminant concentration^{3,4}. Using hydrologic models, Johannes⁴ estimated that 50% of the nitrogen input into the coastal area of Perth, Australia, resulted probably from submarine groundwater discharge; several other recent studies provide indirect evidence that groundwater may be an important nutrient source in several

lakes^{16–18}, as well as along the coasts of Massachusetts¹⁹ and Jamaica²⁰.

Very high rates of phytoplanktonic productivity are found in the Great South Bay²¹ where nitrogen is the primary limiting nutrient⁹. Previous estimates of new inputs of nitrogen to the bay, through runoff and stream flow, appear small in the light of total demands²² but, using Bokuniewicz's⁵ estimate of average daily discharge of groundwater into the bay and assuming 10 μM nitrate near the sediment/water interface, we calculate that groundwater discharge could account for $\geq 20\%$ of the estimate for nitrogen input by runoff²², the other major source of new nitrogen to this lagoon.

The microbiota of shallow coastal sediments are intimately involved in nitrogen cycling in adjacent waters, receiving and metabolizing the rain of organic detritus and regenerating and recycling inorganic nutrients²³. The efflux of nutrients from sediments often supports a large fraction of the algal production in overlying waters²⁴. As well as contributing directly to observed

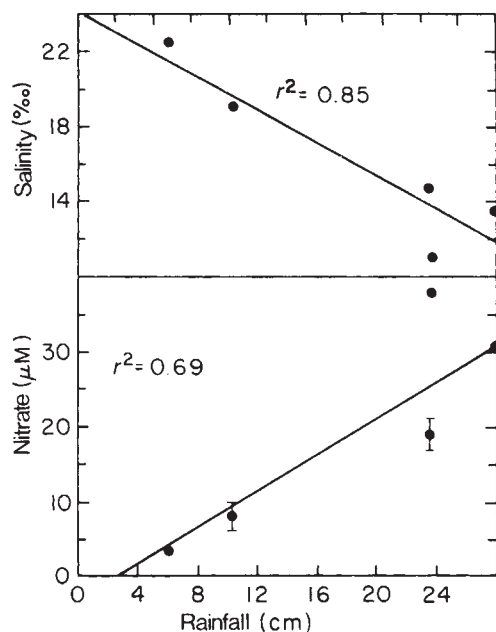


Fig. 4 Correlation of mean interstitial nitrate and salinity (15–40 cm) with rainfall over the previous 30 days. Climatological data for Long Island from the meteorological group of Brookhaven National Laboratories, Rainfall in central Brookhaven township was 3.9 inches (9.8 cm) for November 1982, 2.4 inches (6.1 cm) in December 1982, 11.1 inches (28.2 cm) in April 1983 and 4 inches (10.2 cm) in May 1983.

nutrient fluxes, groundwater nitrate permeating through the sediments may be dissimilated to ammonium, an important route for nitrate consumption in anoxic marine sediments which accounts for 20–80% of the nitrate flux^{25,26}. It is probable that part of the ammonium efflux observed at our site²² arises from nitrate dissimilation in the anoxic zone and, as such, contributes new, as opposed to recycled, input to the bay²⁴.

Nitrate-enriched groundwater effusion through shallow sediments also may have direct effects on the nature of microbial activities in those sediments; bacterial denitrification, the respiratory reduction of nitrate to gaseous nitrogen (N_2 or N_2O)²⁴, has been documented in various coastal sedimentary environments^{25–28}. Denitrification is usually of little consequence in the anaerobic, heterotrophic consumption of organic carbon because of the low NO_3^- concentration in anoxic sediments²⁹. The potential energy gain in coupling NO_3^- reduction to carbon oxidation is considerably greater than that derived from SO_4^{2-} reduction^{23,29}, the predominant respiratory mode of anaerobic marine sediments^{29,30}. It is therefore possible that in regions subjected to persistent nitrate-enriched groundwater flow, denitrification would attain quantitative significance with respect to carbon cycling and stimulated denitrification would serve also to mitigate the effect of groundwater discharge on algal growth in overlying waters. Indeed, in preliminary experiments, we find denitrification in the lower portions of these sediments is inversely correlated with sulphate reduction, correlated with nitrate and apparently limited by the availability of organic substrates.

In addition to high levels of inorganic nitrogen, groundwaters are often contaminated by a spectrum of synthetic organic and metal pollutants^{2,3}. Hence the identification of nitrate derived from groundwaters in coastal sediments suggests this as an additional route that must also be considered for transfer of persistent metal and organic pollutants to marine ecosystems.

Although submarine groundwater discharge as a source of new nitrogen to coastal marine ecosystems has been suggested, it has been neither generally recognized⁴ nor directly measured before; the data described here are the first substantive evidence for this phenomenon in a nearshore coastal environment. We believe that the discharge of nitrate-enriched groundwater through coastal sediments has important implications in coastal

zone management, plankton dynamics, sedimentary geochemistry, microbial ecology and environmental toxicology.

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Declining Phanerozoic background extinction rates: effect of taxonomic structure?

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A decline in the total and per-family extinction rate of marine families^{1,2} during the Phanerozoic has been attributed to progressive improvements in the ecological properties of species. We believe this is not necessarily the case and suggest that the lower family extinction rates may reflect increases in the number of species per family, on the geological time scale, with higher species/family ratios being an inevitable consequence of the increase in species richness³ and the geometry of the branching evolutionary tree⁴. Because species-rich families are more likely to survive the stochastically constant background probabilities of species extinction, the biosphere has gradually accumulated species-rich clades and the total and per-family rates of extinction have consequently declined.

A new compilation of the stratigraphic ranges of fossil groups⁵ has permitted detailed analyses of total¹ and per-family² extinction rates of Phanerozoic families of marine metazoans, which show a statistically significant decline in familial extinction

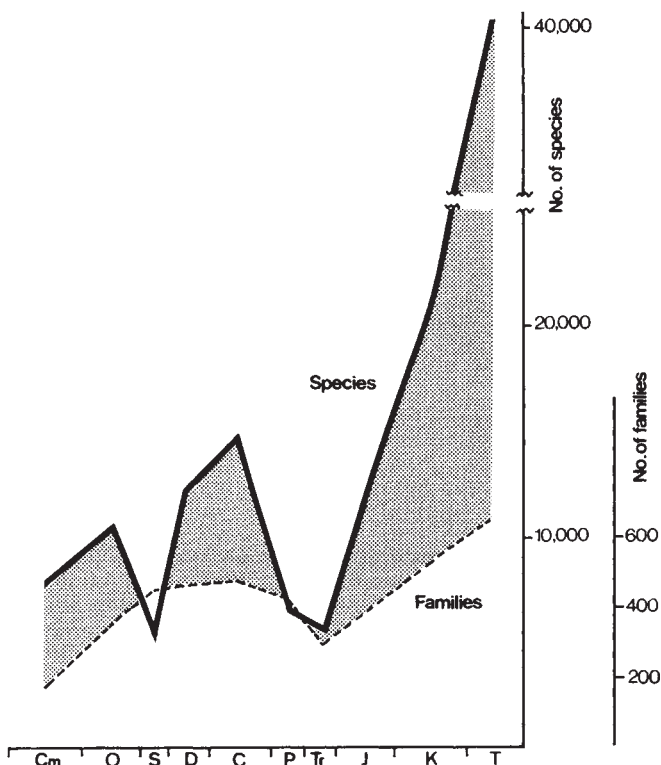


Fig. 1 Diversification of skeletonized marine organisms during the Phanerozoic. Curves show the average number of families¹⁶ (dashed line) and approximate number of species¹⁷ (solid line). Cm, Cambrian; O, Ordovician; S, Silurian; D, Devonian; C, Carboniferous; P, Permian; Tr, Triassic; J, Jurassic; K, Cretaceous; T, Tertiary.

during most of the Phanerozoic. Speculation that this trend represents an increase in the average species fitness on the evolutionary time scale is ostensibly opposed by the existence of two distinct episodes of declining background extinction, defined by Van Valen² (Vendian-early Permian and late Permian-Neogene), who has proposed instead that the trend resulted from minimizing the growth of ecological interference. Whereas the fitness model would require that the 'fitter' species of the Permian be selectively exterminated, the Permo-Triassic extinctions would, in Van Valen's view, serve only to "reset the clock of community evolution".

Extrapolation of such microevolutionary hypotheses to the grand temporal sweep of the fossil record and to higher taxa is attractive but may not be required by the data. The branching of the evolutionary process imposes a taxonomic hierarchy on the history of life, at the higher levels of which evolutionary phenomena may result from properties not affecting the fate of individual species. It is, therefore, appropriate to use this hierarchy in the study of evolutionary patterns.

Palaeontological species counts, although ideal for analyses of taxonomic rates of evolution, are generally unreliable. Typically, numbers of families are used often with the implicit assumption that they track numbers of species without any significant bias. However, there is a variety of palaeontological and neontological evidence that familial diversity does not increase in direct proportion to species diversity (Fig. 1) which has profound consequences for the analysis of evolutionary patterns above the species level.

The post-Silurian Palaeozoic record reveals a regular increase in the number of species per family. The post-Palaeozoic change in taxonomic structure is dramatic, with the increase in species richness exceeding the level of family diversification by a factor of three (that is, species richness has increased 7-fold but family richness only 2.3-fold). Valentine⁶, having independently noted marked increases in the number of genera per family during the Phanerozoic (especially so during the Cretaceous and

Cenozoic), has estimated that the late Phanerozoic increase in species richness outpaced the family diversification by a factor of five. Furthermore, Raup⁷ has reported that the species-family ratio of post-Palaeozoic echinoids has increased, even when the effects of differing species richness within each time interval have been removed (through rarefaction).

The behaviour of species-family ratios in extant faunas also supports our argument that the taxonomic structure of the global biota changed during the Phanerozoic. Sampling studies demonstrate that, although the initial increase in species numbers is matched by an increase in the number of families, the rate of increase of family diversity eventually declines despite the addition of new species⁸, which is simply a consequence of the 'hollow curve' in the distribution of species within higher taxa⁹. Among island biotas, for example, the species-family ratio increases for progressively more species-rich samples^{10,11}. Increasing diversification during the Phanerozoic³ thus suggests a change in the average species-family ratio since the Precambrian.

Because families with many species will resist stochastic extinction better than families with only a few species^{6,12,13}, any long-term shift in the taxonomic structure of the global biota will have far-reaching macroevolutionary effects. Figure 1 shows that post-Triassic species diversification caused a progressive increase in species-family ratios, resulting in faunas composed increasingly of species-rich families that would be relatively resistant to the stochastically constant probabilities of species extinctions that may typify periods of background extinction. A progressive decline in familial extinction rates and probabilities would be inevitable. Sharp increases in species-family ratios are not as apparent in the Palaeozoic record and the decline in extinction rates then may be caused by other factors. However, the quality of the fossil record, particularly at the species level, decreases with increasing age⁸ and accurate estimates of these ratios may be difficult to obtain.

A quantitative assessment of the effects of changing taxonomic structure is frustrated by problems of time series, data selection and data arrangements. The Phanerozoic decline in background extinction rates (excluding the five major mass extinctions) explains only 22% of the total variation¹. When the two-stage decline in probabilities is considered, r^2 values range from 0.56 to 0.60 (ref. 2). These values, however, are based on analyses that include mass extinctions, which, in conjunction with the *ad hoc* partitioning of data points, may have resulted in inflated coefficients of determination.

We do not wish to use these arguments to claim that there has definitely been no 'improvement' in species qualities; we suggest only that the extant analyses of familial extinctions provide insufficient evidence for any improvement. The trends cannot be regarded as mere artefacts of taxonomic structure because species richness may well be a clade property contributing to macroevolutionary survival during the intervals free of mass extinctions. The overall trend of declining background extinction persists despite several episodes of mass extinction¹ during which species-rich families apparently lose their relative resilience¹⁴, which suggests that mass extinctions do not completely reset the macroevolutionary clock: some species-rich families do indeed persist, allowing the marine biota to continue to accumulate taxa resistant to the effects of background species extinction. Thus, we conclude that the global biota owes its present composition to a complex interaction of mass and background extinctions and, because the effects of species extinctions on the evolution of higher taxa vary according to the biota's changing taxonomic structure¹⁵, a hierarchical approach to the analysis of macroevolutionary rates is required.

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Hydrodynamic focusing of motile algal cells

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The swimming direction of algal cells can be guided so that the cells are focused into a concentrated beam. This directed locomotion, or taxis, results from the orientation of the cells' axes by compensating gravitational and viscous torques. It is named gyrotaxis because of this origin. Gyrotaxis includes rheotaxis¹, which is concerned with orientation and locomotion of elongated microorganisms, especially spermatozoa, in fluids with a velocity gradient. I present here a simplified theory of gyrotaxis, together with experimental evidence. A geometrical arrangement is shown whereby the effect can be made the basis of a new method for concentrating and separating motile cells. Unlike standard concentration/separation techniques, the gyrotactic method requires active participation of the cells and can, in principle, distinguish among them on the basis of morphology and swimming behaviour. I also discuss the role of gyrotaxis in the maintenance of naturally occurring descending streams of cells and in bioconvection patterns.

Biflagellated cells such as *Chlamydomonas* or *Dunaliella* are spheroidal. They are shown in Fig. 1 as an idealized sphere of radius a . The flagella are anterior. Their back and forth movement produces a jerky breaststroke²⁻⁴ with mean speed $v_c \ll$

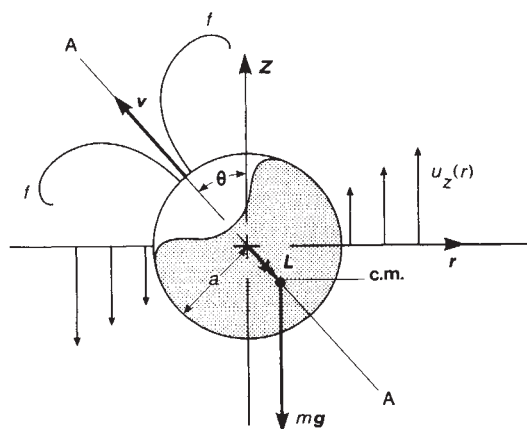


Fig. 1 Idealized spherical algal cell and relative fluid velocity $u_z(r)$. The angle θ increases counterclockwise. The stippling represents the chloroplast which determines the location L of the centre-of-mass (c.m.) relative to the geometrical centre $+$. The flagella f propel the cell along AA with velocity v . The force of gravity produces a clockwise torque $mgL \sin \theta$ around $+$.

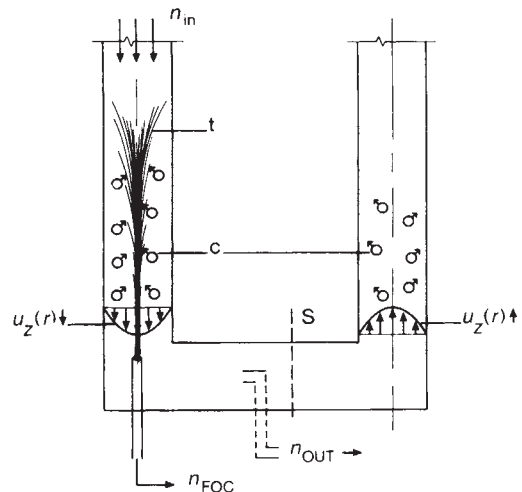


Fig. 2 Apparatus for demonstrating gyrotactic focusing. Vertical cylindrical tubes are connected by a horizontal section, so that cell-containing fluid flows with velocity distribution $u_z(r)$ in both tubes, downwards on the left, upwards on the right. c , The cells' locomotion relative to $u_z(r)$ is indicated by small arrows attached to spheres which represent cells. The cells swim towards the axis in the downwards stream and towards the periphery in the upwards one. t , Focusing cell trajectories in the laboratory system. Schematic conversion of the apparatus to a gyrotactic separation spectrometer is accomplished by a cut and closure at S . Some incoming radially-uniform concentration of cells n_{in} are focused to the concentration n_{FOC} and extracted by an axial pipe. The remaining cells, n_{OUT} , are extracted downstream.

$200 \mu\text{m s}^{-1}$. The cell radius is $3-5 \mu\text{m}$, the density for typical algal cells is $5-10\%$ greater than that of the aqueous medium in which they swim. Relative to the centre of buoyancy or drag of the cell, the centre of gravity is displaced backwards by the vector L . The displacement L is due to the posterior location of relatively heavy organelles such as the chloroplast. The colinear placement of L and the swimming velocity v on the cell axis AA is approximate. The magnitude of L was estimated by microscopic observation of the rotation immobilized cells of *D. tertiolecta* suspended in fluid of the same density as the cells, to prevent sedimentation. L was found to vary from 1 to 5% of the mean cell radius, a . When the cells were allowed to sediment, in pure water, the drag of the rigid flagella increased the rate of reorientation by as little as a few % and as much as a factor of two. If this sedimentation reorientation⁵ occurs in swimming cells, it contributes to the effective magnitude of L . Thus, as a result of the cells' asymmetry, the force of gravity exerts a torque $L \times g = \hat{\phi} mgL \sin \theta$, clockwise in the case illustrated in Fig. 1, where $g = -g\hat{z}$, $\hat{\phi} = \hat{z} \times \hat{r}$, and $(\hat{r}, \hat{\phi}, \hat{z})$ are unit vectors.

A body immersed in a streaming fluid experiences hydrodynamic torque⁶ due to the spatial variation of the fluid velocity $u(r)$. The hydrodynamic and gravitational torques on a sphere sum to

$$T = 8\pi\mu a^3[(\nabla \times u)/2 - \omega] + mL \times g \quad (1)$$

where ω is the angular velocity of the sphere, $\nabla \times u$ is the vorticity, and μ is the viscosity. Nonspherical bodies experience an additional torque due to the rate-of-strain tensor. For swimming cells, there is also a time-varying factor due to the moving flagella. These additional torques will be ignored in the present elementary treatment.

In the absence of directed locomotion such as phototaxis, equation (1) specifies the orientation of the axis AA and, because the cells swim, of their trajectories relative to the velocity field $u(r)$. Due to the small Reynolds number, components of v initially orthogonal to $\nabla \times u$ are rapidly damped away. Setting $T = 0$, equation (1) yields a time-independent cell orientation θ for constant and sufficiently small vorticity. When $u = u(r)\hat{z}$, the

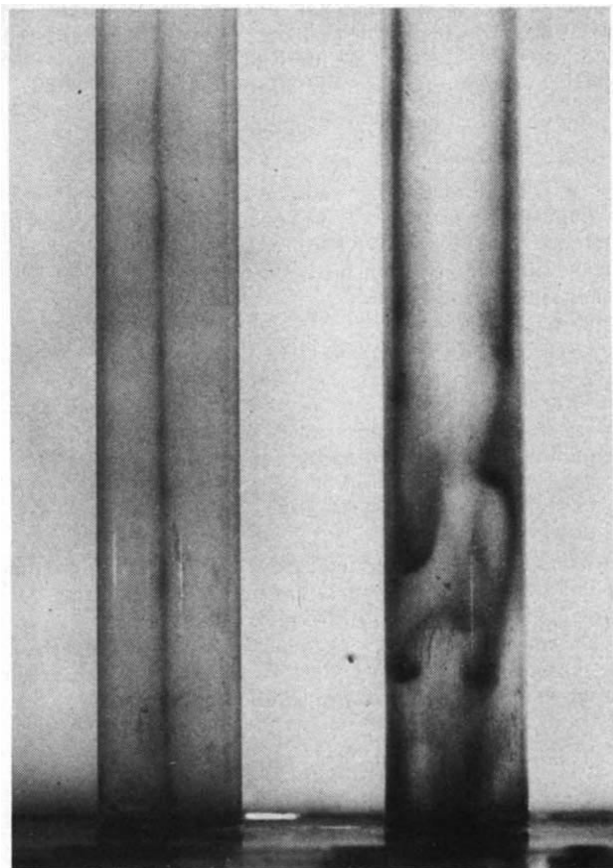


Fig. 3 Demonstration of gyrotactic focusing. Fluid containing the algae *Chlamydomonas nivalis* flows downwards at the left, upwards at the right, as in Fig. 2, causing cells to accumulate axially and peripherally, as predicted. Because the cells' density is greater than the fluid's, high concentration cell accumulations sink. The right-hand side shows the peripherally-sinking plumes, which are narrowed by gyrotactic focusing in their self-generated velocity profile. The cell concentration before focusing is $\approx 10^6$ cells cm^{-3} , $u_0 \approx 0.1 \text{ cm s}^{-1}$, $2R = 1.25 \text{ cm}$ = inside diameter of tubes.

static torque balance gives

$$\sin \theta = (\beta/v_c)(\nabla \times \mu)_\phi \quad (2)$$

where the gyrotactic length $\beta = 4\pi\mu a^3 v_c / mgL$ includes all the parameters describing the cells' motion. For $a = 4 \mu\text{m}$, $v_c = 200 \mu\text{ms}^{-1}$ and $L = 0.03a$, $\beta = 0.05 \text{ cm}$.

For vertically downward Poiseuille flow of the cell-containing fluid in a cylinder of radius R , $\mathbf{u}(r) = -u_0(1 - r^2/R^2)\hat{z}$ and, therefore, $v_c \sin \theta = 2u_0 r \beta / R^2$. Because the stable orientation occurs for $0 \leq \theta \leq \pi/2$, the cell velocity in the laboratory reference frame is

$$\mathbf{c} = -(v_c \sin \theta)\hat{r} + [v_c \cos \theta - u_0(1 - r^2/R^2)]\hat{z} \quad (3)$$

The cells swim towards the axis of the cylinder, across the streamlines of the flow, the vorticity and the acceleration of gravity, a behaviour which is indeed observed. Equation (3) neglects sedimentation because that velocity is much smaller than v_c or u_0 in the situations considered. The time constant for radial influx is $t = R^2/2u_0\beta$. The development of a centrally-focused column of cells in a downward flow characterized by $R = 0.6 \text{ cm}$, $u_0 = 0.1 \text{ cm s}^{-1}$ occurred within 5–10 cm, which corresponds, as one would predict, to a distance of $\sim 2u_0 t$.

When cells which normally exhibit gyrotactic focusing were killed with a small amount of tincture of iodine, there was no radial accumulation. Furthermore, live cells which do not swim, such as *Chlorella*, also do not focus. A more revealing consistency check which tests the fundamental symmetry of the theory uses an upwards Poiseuille flow. Cell-containing fluid

flowed downwards in one leg of a U-tube, as in Fig. 2, and upwards, at the same rate, in the other. For the upwards flow, the sign of u_0 is opposite to that for downflow, and hence $\nabla \times \mathbf{u}$ and $\sin \theta$ also reverse sign. In the upwards flow, cells should, therefore, move away from the axis and accumulate at the periphery of the cylinder. This behaviour is observed and shown in Fig. 3. The demonstration that reversal of $\mathbf{u}$ with respect to g changes the sign of gyrotaxis proves the theory and eliminates the possibility that the effect is a general consequence of flowing suspended motile organisms through an arbitrarily oriented pipe.

Gyrotaxis provides a new method for separating and concentrating cells. The components of equation (3) can be integrated to yield cell trajectories. From these trajectories, or from the steady-state cell conservation equation

$$u_z \frac{\partial n}{\partial z} = -\frac{1}{r} \frac{\partial}{\partial r} r \left(-\beta n \frac{du_z}{dr} \right) \quad (4)$$

one can show that

$$-\frac{4\beta}{R^2} z = \ln \frac{n(r, z)}{n_0} + \frac{r^2}{R^2} \left(1 - \frac{n(r, z)}{n_0} \right) \quad (5)$$

where n_0 is the cell concentration entering at $z = 0$, and $n(r, z)$ is the downstream concentration. If, in equation (4), a diffusive term $-D\partial n/\partial r$, which idealizes random-cell swimming components or cell-cell collisions, is added to the gyrotactic cell flux, one obtains

$$n(r) = \text{constant} \times \exp(-\beta u_0 r^2 / DR^2) \quad (6)$$

as $4\beta|z|/R^2 \gg 1$. Equations (4)–(6) assume, for simplicity, that the $\cos \theta$ term in equation (3) can be ignored, that is $v_c \ll u_0$.

Equations (5) and (6) determine the design of an apparatus for concentrating cells and separating them from each other on the basis of morphology and motile behaviour, that is, on the spectrum of β and D . Figure 2 illustrates the apparatus, called a gyrotactic separation spectrometer. Cells contained in a culture which flows down the left part of the U-tube, or a separate single vertical tube, are centrally concentrated at a rate which depends on β and D . The focused concentration, n_{FOC} , is extracted near the tube axis and thereby separated from the unfocused cells which descend near the tube's periphery. A gyrotactic separation spectrometer has been built and used to separate *Chlamydomonas* from *Chlorella* and cold-stressed *C. reinhardtii* CW 15, which exhibit motility defects, from similar cells which are normal. These experiments, carried out with the cooperation of G. J. Morris and N. C. Pennick, will be described more fully elsewhere. Initial cell concentrations n_0 ranged from 10^4 to 10^7 cells cm^{-3} . Focal concentrations up to 8.7 times n_0 were achieved in one pass through the apparatus.

The gyrotactic phenomenology and theory described so far have ignored the modification of local average fluid density by the presence of the cells. The excess density due to a concentration n of cells having volume W and density $\Delta\rho$ higher than the suspending medium's is $Wn\Delta\rho$. For example, for $n = 10^7 \text{ cm}^{-3}$, $W = 10^{-10} \text{ cm}^3$ and $\Delta\rho = 0.1 \text{ g cm}^{-3}$, the excess density is $10^{-4} \text{ g cm}^{-3}$, sufficient to produce a substantial sinking velocity of a cell cluster. A sinking region produces a fluid velocity field which guides further gyrotactic accumulation transversely into it. This positive feedback, or cooperative self accumulation, generates or maintains the sharply focused coherent descending plumes of cells frequently observed in dense algal cultures. The effect may occur in nature, during algal blooms, and it may be observed in the vertical U tube shown in Fig. 3. The cells which have accumulated at the periphery of the up-flow spontaneously form sinking plumes. The focused beam of cells in the down-flowing stream is actually sinking at a greater rate than u_0 , due to its excess weight. The self-accumulation effect is associated with a characteristic instability which shows up in Fig. 3 as a series of bulbous expansions of the beam of algae. The instability is insignificant at the lower n_0 values employed in the gyrotactic separations cited above.

The buoyant convection due to gyrotaxis is an important factor in the generation of regular patterns of fluid convection and cell concentration enhancement observed in concentrated algal cultures⁷. It had been thought^{8,9} that these patterns were entirely caused by the generation of adverse density gradients due to the up-swimming of the cells. The importance of gyrotaxis in the maintenance of steady patterns can be inferred from the characteristic appearance of vertical plumes of cell concentration at the bottom of a culture, but with no connection of these plumes to the upper regions of the cell-containing fluid. Note that it has so far not been possible to achieve gyrotactic focusing of *Tetrahymena pyriformis*, one of the principal organisms considered⁹ in the 'old theory' of pattern formation. Therefore, the old theory is probably entirely applicable to that case, and possibly to ciliates generally.

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Gestation period, neonatal size and maternal investment in placental mammals

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Two factors complicate the analysis of scaling relationships between neonatal size or gestation period and maternal size in mammals: (1) a fundamental distinction between species with poorly-developed (altricial) neonates and those with well-developed (precocial) neonates; (2) interaction between all three variables. We show here that altricial and precocial mammals can be clearly distinguished by gestation period relative to maternal weight. Separate analysis of the three variables for altricial and precocial mammals yields more appropriate allometric formulae which can in turn be related to a standard fetal growth formula derived from single-species studies. Hence, an overall equation can be derived across species which links neonatal weight to a combined function of gestation period and a fetal growth parameter. The latter, which provides a measure of maternal investment, is a constant for each species but varies allometrically with maternal weight across species¹. Analysis of the fetal growth parameter across species shows that altricial mammals generally have a higher maternal investment for a given gestation period and maternal weight than precocial mammals.

Studies of fetal development as a function of time in individual mammal species²⁻⁵ have yielded the following standard fetal growth formula; this can be derived theoretically⁵ and has received strong empirical confirmation:

$$W = a(t - t_0)^3 \quad (1)$$

where W is fetal weight at gestational age t , t_0 is the 'lag' phase following conception and a is the specific fetal growth velocity. As noted by Payne and Wheeler⁵, this formula automatically yields the following relationship between neonatal weight (W_N) and gestation period (G):

$$W_N = a(G - t_0)^3 \quad (2)$$

Because t_0 is small relative to G and can be regarded as an approximately constant proportion of G (estimated at ~20%)⁵, this equation can be simplified for comparative purposes to:

$$W_N = a'G^3 \quad (3)$$

where a' is the approximated fetal growth factor. Although some degree of size-dependency of t_0 has been reported⁶, this does not significantly affect the following calculations.

In an entirely different approach, involving comparisons across species, neonatal weight (W_N) has been related to maternal weight (W_M) by empirical allometric formulae of the type⁶⁻¹⁴:

$$W_N = kW_M^\alpha \quad (4)$$

where k is the neonatal scaling coefficient and α the neonatal scaling exponent. Some authors have calculated W_N as the individual neonate weight, whereas others have calculated it as the total litter mass (that is, individual neonate weight $\times$ average litter size). For large samples of placental mammal species, the scaling exponent for individual neonate weight has been reported¹¹⁻¹³ as 0.89-0.94 and the exponent for total litter mass^{7-9,11-14} as 0.77-0.84. As equations (1), (2) and (3), derived from fetal growth studies, relate to individual neonate weight, only this parameter will be considered here.

In the second interspecific approach, several authors^{12,14-18} have studied the scaling relationship between gestation period and maternal body weight, deriving empirical allometric equations of the following form:

$$G = cW_M^\beta \quad (5)$$

where c is the allometric coefficient and β the scaling exponent. In this case, observed values of the scaling exponent (β) are typically quite low. For placental mammals overall, a value of approximately 0.25 has been reported^{12,16}, whereas for individual orders of mammals a still lower value of about 0.15 has been reported^{6,15,17}.

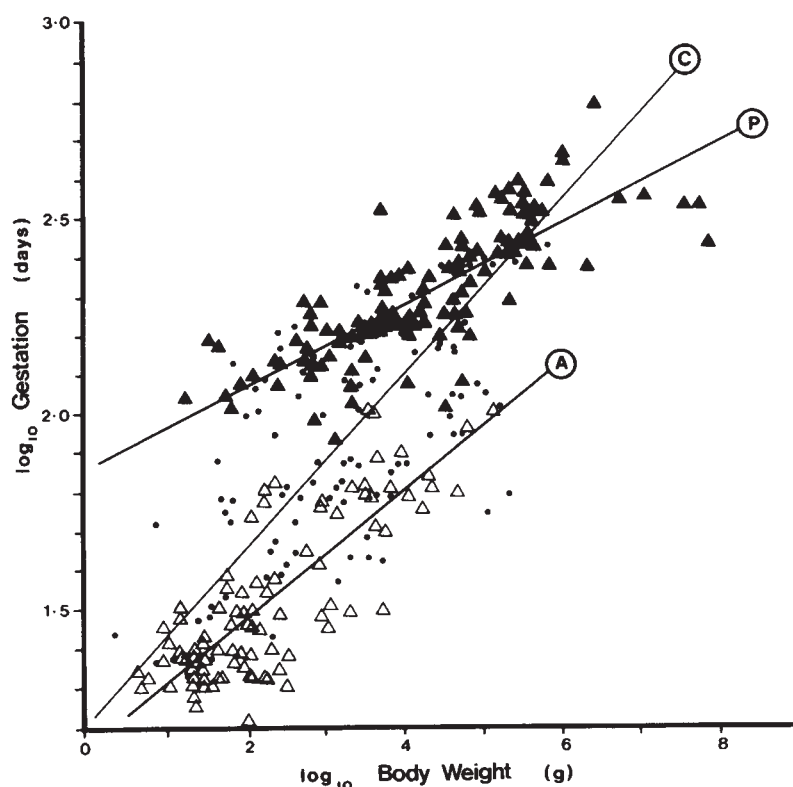
Both interspecific scaling formulae cited (equations (4) and (5)) have two limitations. First, the simple bivariate approach cannot take account of the complex interaction between W_M , G and W_N . For instance, it is likely that for a given value of G , the weight of the neonate (W_N) is likely to depend, at least to some extent, on maternal body size (W_M). Second, it is essential to take into account the state of the neonate at birth because, other things being equal, a poorly-developed neonate should require a shorter gestation period than a well-developed neonate. Many studies, notably those of Portmann¹⁹, have shown that the neonates of placental mammals fall clearly into two categories: altricial (poorly-developed offspring with eyes and ears closed at birth, virtually no hair on the body and typically born in multiple litters) and precocial (well-developed offspring with eyes and ears open at or soon after birth, hair coat well developed, typically born as singletons). Most orders of mammals are characterized by a single type of neonate; insectivores, rodents and carnivores generally have altricial offspring, while

Table 1 Results of bivariate allometric analyses

Group	Gestation period vs. maternal weight				Neonatal weight vs. maternal weight				Fetal growth factor vs. maternal weight			
	<i>n</i>	β	95% c.l.	<i>r</i>	<i>n</i>	α	95% c.l.	<i>r</i>	<i>n</i>	γ	95% c.l.	<i>r</i>
Complete sample	394	0.22	0.20–0.23	0.80	345	0.93	0.90–0.95	0.97	291	0.33	0.28–0.38	0.60
Precocial mammals*	125	0.11	0.09–0.12	0.79	130	0.91	0.88–0.94	0.98	102	0.59	0.53–0.65	0.90
Altricial mammals*	97	0.17	0.14–0.19	0.80	73	0.75	0.71–0.80	0.97	70	0.28	0.20–0.37	0.61
Primates	66	0.13	0.10–0.16	0.73	74	0.85	0.80–0.91	0.93	53	0.54	0.44–0.65	0.82
Simian primates†	44	0.10	0.07–0.12	0.77	51	0.77	0.72–0.83	0.97	33	0.50	0.39–0.62	0.85
Strepsirrhine primates‡	22	0.10	0.00–0.21	0.41	20	0.72	0.65–0.80	0.98	20	0.60	0.25–1.10	0.60
Artiodactyls	64	0.13	0.10–0.17	0.64	42	0.89	0.79–1.00	0.93	41	0.54	0.35–0.78	0.77
Carnivores	56	0.11	0.07–0.16	0.59	54	0.76	0.65–0.89	0.86	47	0.56	0.44–0.68	0.80
Myomorph rodents§	76	0.09	0.04–0.13	0.40	46	0.64	0.54–0.74	0.89	44	0.41	0.25–0.60	0.58
Hystricomorph rodents	27	0.14	0.05–0.24	0.55	27	0.89	0.80–1.00	0.97	24	0.68	0.34–1.16	0.62

n, Number of animals studied; c.l., confidence limits; *r*, correlation coefficient. * See text for definition; † monkeys, apes and man; no reliable data for tarsier gestation; ‡ lemurs and lorises; § typically altricial; || exceptional among rodents in being typically precocial.

Fig. 1 Logarithmic plot of gestation period (days) against maternal body weight (g) for a sample of 394 placental mammal species. Best-fit lines (major axes) have been determined separately for altricial mammals (line A, open triangles), precocial mammals (line P, solid triangles), and for the complete sample (line C). Black dots indicate mammals intermediate between clearly altricial and clearly precocial species (see text for definition).



artiodactyls, perissodactyls, cetaceans and primates generally have precocial offspring. However, exceptions and intermediate cases occur, and it is commonly implied that there is a smooth continuum from altricial to precocial types among placental mammals.

For purposes of analysis, three groups of placental mammals can be defined: (1) clearly precocial mammals, in which the litter size is less than 1.5 and the eyes are already open at birth; (2) clearly altricial mammals, in which the litter size is three or more and the eyes do not open until at least 5 days after birth; (3) intermediate cases, with litter sizes between 1.5 and 3, with eyes opening between birth and 5 days of age. Examination of the relationship between gestation period and maternal body weight using a logarithmic bivariate plot (Fig. 1) reveals a sharp separation between clearly precocial and clearly altricial mammals. This is a particularly convincing example of the presence of major allometric 'grade' distinctions²⁰. The allometric exponent values for precocial and altricial mammals are quite similar (Table 1), giving an average of $\beta = 0.14$ (see equation (5)). A best-fit line applied to the whole sample of placental mammals has a markedly steeper slope of 0.22 (Fig. 1, Table

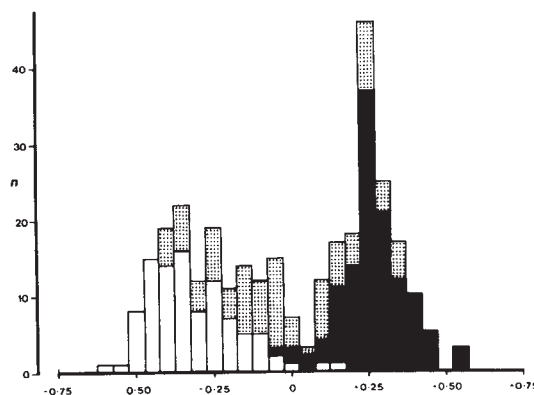


Fig. 2 Histogram of residuals, relative to an overall best-fit line of fixed slope 0.10, for the species shown in Fig. 1. Note the almost complete separation between clearly altricial mammals (white boxes) and clearly precocial mammals (solid boxes). Intermediate species (stippled boxes) do not obliterate the bimodal pattern.

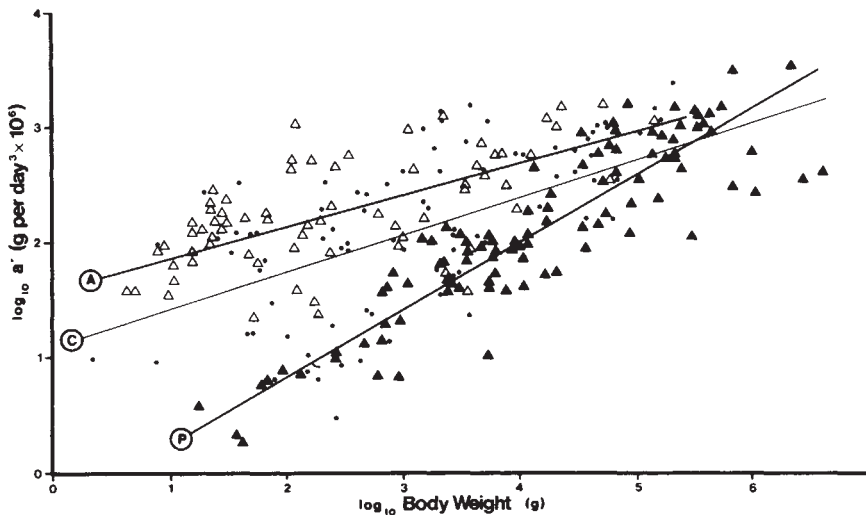


Fig. 3 Logarithmic plot of fetal growth factor values (a') against maternal body weight (g) for a sample of 291 placental mammal species. Symbols as in Fig. 1.

1), close to the value previously reported; but this can now be seen to result from confusion of separate grades. However, even within the major grades of precocial and altricial mammals there are more subtle grade distinctions, and analysis of separate taxonomic groups (Table 1) reveals an average exponent value of 0.11 (range 0.09–0.14).

Because of this inherent problem of grade confusion, an alternative approach was applied, involving iteration of exponent values, to obtain the best separation of clearly precocial and clearly altricial mammals. This was achieved with a line of fixed slope 0.10, as reflected by the distribution of residuals of individual points relative to that line. A histogram of residuals (Fig. 2) shows the bimodal separation of precocial and altricial mammals (as defined above) and also shows that inclusion of 'intermediate' mammals does not obliterate the bimodal pattern. The distinction between precocial and altricial mammals is hence very pronounced; with a fixed exponent value of $\beta = 0.10$, a typical precocial mammal has a gestation period almost four times longer than a typical altricial mammal of the same body size. For comparative purposes, therefore, equation (5) can be rewritten as follows, with the proviso that the value of the allometric coefficient (c) differs sharply between altricial and precocial mammals:

$$G = cW_M^{0.1} \quad (6)$$

Similar considerations apply to the relationship between individual neonate weight and maternal weight. Because precocial mammals have markedly longer gestation periods than altricial mammals, relative to body size, it follows from equation (3) that, other things being equal, they must also produce much larger neonates. Precocial mammal species generally have greater adult body weights than altricial mammals (Fig. 1), so a single best-fit line for neonatal weight against maternal weight for all placental mammals will have an artificially high slope, reflecting grade confusion, just as in the case of gestation period²¹. The neonatal scaling exponent value of $\alpha = 0.93$ (see equation (4)) for the overall sample of placental mammals (Table 1), which agrees closely published values^{7,10–12}, is accordingly misleading. Analysis of individual taxonomic groups (Table 1) suggests that a value of $\alpha = 0.8$ would be more appropriate. Equation (4) can, therefore, be rewritten as follows, to reflect empirical findings:

$$W_N = kW_M^{0.8} \quad (7)$$

An apparent paradox arises⁶ when equations (3) and (6) are combined to yield an alternative formula relating neonatal weight to maternal weight:

$$W_N = a'G^3 = a'(cW_M^{0.1})^3$$

$$W_N = a'c^3W_M^{0.3} \quad (8)$$

It has commonly been assumed that the fetal growth factor (a') is constant not only within species but also between species, at least for taxonomic groups containing species with broadly comparable habits²². However, the apparent conflict between equations (7) and (8) with respect to the exponent values (0.8 compared with 0.3) arises because the fetal factor actually varies with maternal body size across species¹. From these two equations, it is possible to derive a predicted allometric relationship between a' and maternal weight:

$$W_N = kW_M^{0.8} = a'c^3W_M^{0.3}$$

Hence

$$a' = \frac{k}{c^3} W_M^{0.5} \quad (9)$$

In fact, the fetal growth factor reflects the differential capacity for investment of fetal growth exhibited by mammalian mothers of different body sizes when other things are equal. Equation (3), can therefore, be rewritten as a product of two allometric functions of maternal size, a maternal investment function (fetal growth factor scaling exponent γ) and a development time function (gestation scaling exponent β):

$$W_N \propto W_M^\gamma (W_M^\beta)^3 \quad (10)$$

Allometric variation in the fetal growth factor in relation to maternal weight can be examined directly following a rearrangement of equation (3):

$$a' \propto \frac{W_N}{G^3} \quad (11)$$

Values for (W_N/G^3) for individual species can be plotted against maternal weight (Fig. 3) and Table 1 shows that empirical values for the scaling exponent (γ) relating fetal growth factor to maternal body weight are reasonably close to the value of 0.5

Table 2 Results of bivariate allometric analyses for neonatal body weight versus maternal body weight in cases where gestation period is relatively constant

Group	n	Exponent value	95% c.l.	r
Domestic dog breeds	8‡	0.56‡	—	0.97‡
<i>Felis</i> spp.	8	0.73	0.45–1.11	0.92
<i>Panthera</i> spp.	6	0.50	0.10–1.10	0.84
<i>Peromyscus</i> spp.	11	0.61	0.37–0.92	0.87
<i>Cercopithecus</i> spp.*	7	0.61	0.15–1.40	0.79
<i>Macaca</i> spp.†	7	0.35	0.12–0.61	0.86

* Including *Miopithecus*; † Leutenegger¹⁰ cited an exponent value of 0.49 for seven macaque species; his data were included here in enlarged samples used to calculate averages for each species; ‡ data from J. K. Kirkwood (personal communication).

expected from equation (9). For individual taxonomic groups of mammals, the average value of γ indicated by Table 1 is approximately 0.55. The predicted value of γ can also be tested by considering mammal groups in which there is variation in maternal body weight without concomitant variation in gestation period (thus eliminating the development time function from equation (10)). This is usually true of individual genera among placental mammals and it is also true of domesticated species, such as the domestic dog (J. K. Kirkwood, personal communication), for which artificial breeding has generated a wide range of adult body sizes across breeds without any significant variation in gestation period. Table 2 shows that for a variety of such cases, the average value of γ determined is again close to 0.55. Thus, overall there is good agreement between the predicted value of γ (0.5) from equation (10) and empirical values (average ~ 0.55) determined both with and without interspecific variation in gestation period. Leutenegger¹⁰ noted the lower exponent value for intrageneric scaling of neonatal weight to maternal weights in macaques and linked this to variation in scaling of brain weight to body weight according to taxonomic level in mammals. However, the latter is probably a very distinct phenomenon related to early attainment of final brain weight

in ontogeny and differential selection on body weight during the early stages of adaptive radiation^{23,24}.

It is clear from Fig. 3 that altricial mammals generally exhibit higher values of the fetal growth factor relative to maternal body weight, although the best-fit lines for altricial and precocial mammals tend to converge at higher maternal weights, suggesting a limitation on maternal investment in all large mammals. Thus, relative to maternal weight, altricial mammals are characterized by a combination of short development times (gestation periods) and high maternal investment levels (fetal growth factors), in contrast to precocial mammals. Hence, the marked dichotomy between altricial and precocial mammals indicated in Figs 1–3 reflects a divergence in overall development patterns and is, therefore, directly relevant to the interpretation of mammalian life history strategies.

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Differentiation of a bipotential glial progenitor cell in single cell microculture

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Although it is known that most cells of the vertebrate central nervous system (CNS) are derived from the neuroepithelial cells of the neural tube (reviewed in ref. 1), the factors determining whether an individual neuroepithelial cell develops into a particular type of neurone or glial cell remain unknown. A promising model for studying this problem is the bipotential glial progenitor cell in the developing rat optic nerve; this cell differentiates into a particular type of astrocyte (a type-2 astrocyte²) if cultured in 10% fetal calf serum (FCS) and into an oligodendrocyte if cultured in serum-free medium³. As the oligodendrocyte–type-2 astrocyte (0-2A) progenitor cell can differentiate along either glial pathway in neurone-free cultures^{2,4}, living axons clearly are not required for its differentiation, at least *in vitro*. However, the studies on 0-2A progenitor cells were carried out in bulk cultures of optic nerve, and so it was possible that other cell–cell interactions were required for differentiation in culture. We show here that 0-2A progenitor cells can differentiate into type-2 astrocytes or oligodendrocytes when grown as isolated cells in microculture, indicating that differentiation along either glial pathway *in vitro* does not require signals from other CNS cells, apart from the signals provided by components of the culture medium. We also show that single 0-2A progenitor cells can differentiate along either pathway without dividing, supporting our previous studies using ³H-thymidine³ and suggesting that DNA replication is not required for these cells to choose between the two differentiation programmes.

We have shown previously that cells of the 0-2A lineage in suspensions and cultures of the developing optic nerve can be identified by means of three antibodies: anti-galactocerebroside (GC), which reacts specifically with the oligodendrocytes⁵, anti-glial fibrillary acidic protein (GFAP)⁶, which reacts specifically with the astrocytes^{5–7}, and the A2B5 monoclonal antibody⁸, which recognizes specific gangliosides⁸ and helps to identify two different types of astrocyte². The 0-2A progenitor cells are A2B5⁺, GC[–], GFAP[–] (ref. 3). When cultured in 10% FCS they rapidly acquire GFAP to become A2B5⁺, GC[–], GFAP⁺ type-2 astrocytes^{2,3,9}; when cultured in serum-free medium (or medium containing <1% FCS, our unpublished observations), they rapidly acquire GC (and more slowly lose A2B5)⁴ to become GC⁺, GFAP[–] oligodendrocytes^{3,9}. As 0-2A cells typically have a process-bearing morphology in culture, they can usually be distinguished from other cells in optic nerve cultures—mainly type-1 astrocytes and meningeal cells, most of which have a fibroblast-like morphology (and are A2B5[–])—by phase-contrast microscopy.

To study the differentiation of individual 0-2A progenitor cells to type-2 astrocytes, cell suspensions were prepared from optic nerves of 7-day-old Sprague-Dawley rats using trypsin, collagenase and EDTA as previously described⁹. At this age, 20–25% of the cells in optic nerve cell suspensions are A2B5⁺, GFAP[–], GC[–] 0-2A progenitor cells, but no A2B5⁺, GFAP⁺ type-2 astrocytes are found as these first appear *in vivo* 8–10 days after birth (R. H. Miller, S. David, R. Patel, E. Abney and M.C.R., in preparation). A dilute suspension of cells in L15 medium (Flow Laboratories) containing 10% (v/v) FCS (L15-10) was put in a 5-cm plastic, bacteriological Petri dish and viewed in a Leitz Diavert inverted microscope. Single cells were picked up in a sterile hand-pulled 10- μ l micropipette using mouth suction and transferred to single wells of a Terasaki microculture plate (Falcon), previously coated with poly-L-lysine (40 μ g ml^{–1} in water overnight) and containing 10 μ l of L15-10. The plates were incubated at 37°C in air for 30 min to allow the cells to adhere, and then the medium was changed to

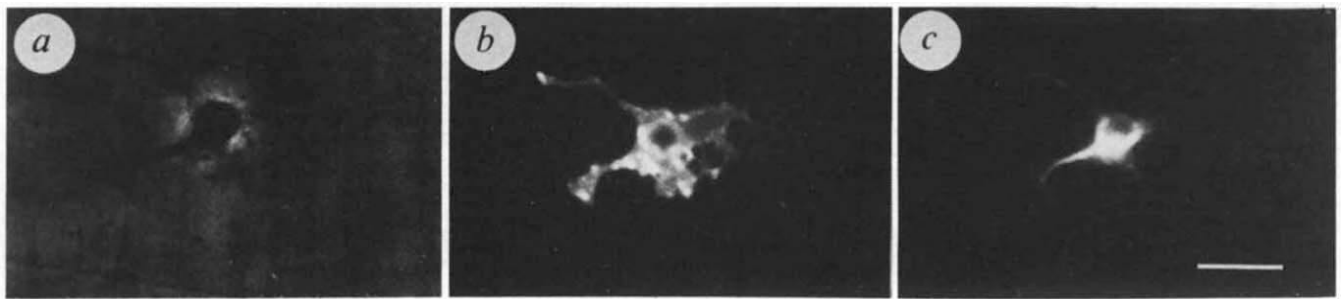


Fig. 1 An A2B5⁺, GFAP⁺ type-2 astrocyte that developed in a single-cell microculture of 7-day optic nerve after growing for 48 h in DMEM-10 and 50% (v/v) conditioned medium from a bulk culture of 7-day optic nerve cells. The cell was visualized by phase-contrast in an inverted microscope (a) and in a conventional fluorescence microscope to show A2B5 (b) and GFAP (c). Scale bar, 20 μ m. The cell was labelled with A2B5 antibody and anti-GFAP serum as described in Table 1 legend. The conditioned medium was prepared as described in Table 1 legend.

Dulbecco's modified Eagle's medium (DMEM) containing 10% or 20% (v/v) FCS and 50 μ M 2-mercaptoethanol (2-ME) (DMEM-10 or DMEM-20). The wells containing single cells were noted and the plates were incubated for 2 days at 37 °C in 5% CO₂. At 24 and 48 h, each cell was checked to see if it had divided or died. At 48 h, wells containing process-bearing cells were double-labelled by indirect immunofluorescence with monoclonal A2B5 (ref. 8) or anti-GC (ref. 10) antibodies and with anti-GFAP (ref. 11) serum as described in Table 1 legend.

Approximately 65% of the cells plated adhered to the microwells by 30 min and more than 80% of these survived for 48 h. Although A2B5⁺ cells constituted only 20–25% of the cells in the original suspension, more than 50% of the cells present at 48 h were process-bearing and A2B5⁺, probably because 0-2A progenitor cells were preferentially selected during micromanipulation. Whereas <20% of the A2B5⁺, process-bearing cells became GFAP⁺ in 10% FCS, >60% became GFAP⁺ in 20% FCS (Table 1), even though the survival, appearance and proliferation rate of these cells were similar in both conditions (not shown). In 20% FCS, 55–60% of the cells divided, but of those which did not, more than 40% became GFAP⁺. Although this indicates that 0-2A progenitor cells can differentiate into type-2 astrocytes *in vitro* without undergoing cell division, the proportion of cells expressing GFAP was higher in the population that had undergone division (Table 1). When 50 process-bearing cells were incubated with anti-GC antibody in cultures maintained in DMEM-10 for 48 h, all were GC⁺ (not shown).

We found previously that >90% of the A2B5⁺ cells in 7-day optic nerve become GFAP⁺ within 48 h in bulk cultures growing in DMEM-10⁹. The present finding—that less than 20% of such cells become GFAP⁺ in single-cell cultures in DMEM-10—suggests that cell-cell interactions in bulk cultures enhance the FCS-induced differentiation of 0-2A progenitor cells into type-2 astrocytes. Conditioned DMEM-10 medium from bulk cultures of 7-day optic nerve markedly increased the proportion of both divided and non-divided A2B5⁺ cells that became GFAP⁺ in single-cell cultures compared with those in fresh DMEM-10 (Table 1), and the intensity of anti-GFAP staining was also increased (Fig. 1). This observation suggests that soluble factors may mediate these putative cell-cell interactions in bulk cultures.

We next studied the differentiation of individual 0-2A progenitor cells to oligodendrocytes using cell suspensions prepared from 1-day rat optic nerve. At this age, ~20% of the cells are A2B5⁺, GFAP⁺, GC⁺ 0-2A progenitor cells, but only rare GC⁺ oligodendrocytes are found (R. H. Miller, S. David, R. Patel, E. Abney and M.C.R., in preparation). Single cells were cultured in microwells for 48 h as described above, except that they were maintained in a modified³ Bottenstein and Sato¹² (B-S) medium (see Fig. 2 legend) containing 2-ME and 0.5% (v/v) FCS (cells survived better in 0.5% FCS than in serum-free B-S medium). Approximately 75% of the cells that adhered to the culture wells at 30 min survived for 48 h, and about 20% of these were process-bearing cells. We found that 55% (36/65) of the process-bearing cells studied at 48 h were GC⁺ (Fig. 2), a similar propor-

tion to that seen in bulk cultures⁹. Although most of these cells had divided, 76% (13/17, combined results of three experiments) of those that did not divide were GC⁺. When single cells were cultured in B-S medium plus 0.5% FCS for 2 days and then switched to DMEM-10 for 3 days, most of the process-bearing cells were still GC⁺, GFAP⁺ (not shown), suggesting that differentiation along the oligodendrocyte pathway is not reversible after 2 days in these conditions, in agreement with results in bulk cultures⁹. While none of the process-bearing cells cultured for 48 h in B-S medium plus 0.5% FCS were GFAP⁺, more than 60% of such cells cultured in DMEM-20 for 48 h were GFAP⁺ (30% of which had not divided) and none were GC⁺ (not shown).

We showed previously that when 0-2A progenitor cells in bulk cultures were switched from DMEM-10 to serum-free B-S medium after 2 days (but not after 3 days), they altered their developmental course and differentiated into GC⁺ oligodendrocytes⁹. Most of these 'switched' cells expressed both GC and GFAP for several days following the medium change⁹. To deter-

Table 1 0-2A progenitor cells from 7-day optic nerve differentiate into type-2 astrocytes in single-cell microcultures containing 10% or 20% FCS

Culture medium	Total A2B5 ⁺ process-bearing cells that are GFAP ⁺	Non-divided A2B5 ⁺ process-bearing cells that are GFAP ⁺
DMEM + 10% FCS	23/147 (16%)	8/60 (13%)
DMEM + 20% FCS	82/133 (62%)	20/48 (42%)
DMEM + 10% FCS + conditioned medium	92/143 (64%)	18/43 (42%)

Cells were cultured as single cells in microwells as described in the text. After 48 h, the process-bearing cells were labelled with monoclonal A2B5 antibody⁸ (ascites fluid, diluted 1:500) as previously described², followed by goat anti-mouse immunoglobulin coupled to rhodamine (G-anti-Mlg-Rd, Cappel, diluted 1:100). Incubations were done in the dark as the cells seemed to survive better. After washing, the cells were fixed in acid-alcohol and labelled with rabbit anti-GFAP serum¹¹ (1:200) followed by sheep anti-rabbit immunoglobulin coupled to fluorescein (S-anti-Rlg-FI, Wellcome, diluted 1:100). The wells were then filled with phosphate-buffered saline (PBS) containing 0.1% (w/v) *p*-phenylenediamine to prevent fluorescence fading¹⁴ and the cells were examined through the bottom of the Terasaki plate in a Zeiss Universal fluorescence microscope using a $\times 40$ phase-contrast objective and photographed using Ektachrome colour slide film rated at 400 ASA. Because it was difficult to see the cells through the bottom of the Terasaki plate by conventional phase-contrast microscopy, each cell was also examined in an inverted Leitz Diavert microscope. The numbers represent the combined results of six different experiments. Conditioned medium was taken from bulk cultures of 7-day optic nerve cells growing in 2 ml of DMEM-10 for 3 days in poly-L-lysine-coated 35-mm Nunc tissue culture dishes. Approximately 30,000 cells were initially plated in each dish. The medium was collected, centrifuged at 1,000g for 10 min and mixed 50:50 with fresh DMEM-10.

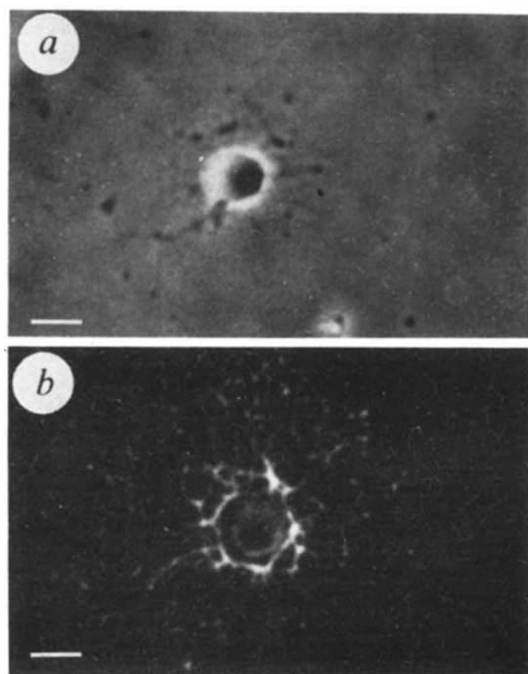


Fig. 2 A GC⁺ oligodendrocyte that developed in a single-cell 1-day optic nerve microculture after 48 h in B-S medium containing 0.5% FCS and 50 μ M 2-ME. The cell was examined by phase contrast in an inverted microscope (a) and in a conventional fluorescence microscope to show GC (b). The cell was cultured in a microwell as described in the text. It was maintained in a modified Bottenstein and Sato medium¹² containing insulin, transferrin, albumin, progesterone, putrescine, selenium, thyroxine and triiodothyronine as previously described³, as well as 0.5% (v/v) FCS and 50 μ M 2-mercaptoethanol. After 48 h, it was labelled with monoclonal anti-GC antibody (ascites fluid, diluted 1:200) followed by G-anti-Mlg-Rd and, after fixation, with anti-GFAP antiserum followed by S-anti-Rlg-F1 as described in Table 1 legend. No staining with anti-GFAP was seen (not shown). Scale bar, 20 μ m.

mine if this switch in developmental course required cell division, we cultured single 7-day optic nerve cells in DMEM-20 for 2 days and then switched them to B-S medium for 3 days. Most of the process-bearing cells that did not divide during this period were both GC⁺ and GFAP⁺ (not shown), suggesting that newly formed type-2 astrocytes can switch their developmental course without dividing.

Our conclusions are as follows. First, 0-2A progenitor cells can differentiate *in vitro* into either type-2 astrocytes or oligodendrocytes without dividing. Moreover, they can switch from the astrocyte to the oligodendrocyte pathway of development after 1 or 2 (but not 3) days in culture⁹ without dividing. These findings suggest that DNA replication is not always required for vertebrate cells to choose between two differentiation programmes as suggested by the quantal mitosis hypothesis¹³. Second, although it is highly likely that cell-cell interactions influence which differentiation pathway individual 0-2A progenitor cells follow during normal development *in vivo*, the differentiation of these cells *in vitro* into either oligodendrocytes or type-2 astrocytes does not require the presence of other cells. On the other hand, signals provided by components in the culture medium, such as those in FCS, probably substitute for signals from other cells. Third, FCS acts directly on the 0-2A progenitor cell to induce it to differentiate into a type-2 astrocyte. Fourth, cell-cell interactions in bulk cultures of optic nerve enhance the effect of FCS in inducing type-2 astrocyte differentiation, probably through soluble mediators. Further studies will be required to determine the nature and source of these mediators, and to discover the cellular and molecular mechanisms controlling the differentiation of 0-2A progenitor cells during normal development.

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Regulation of glutamate receptor binding by the cytoskeletal protein fodrin

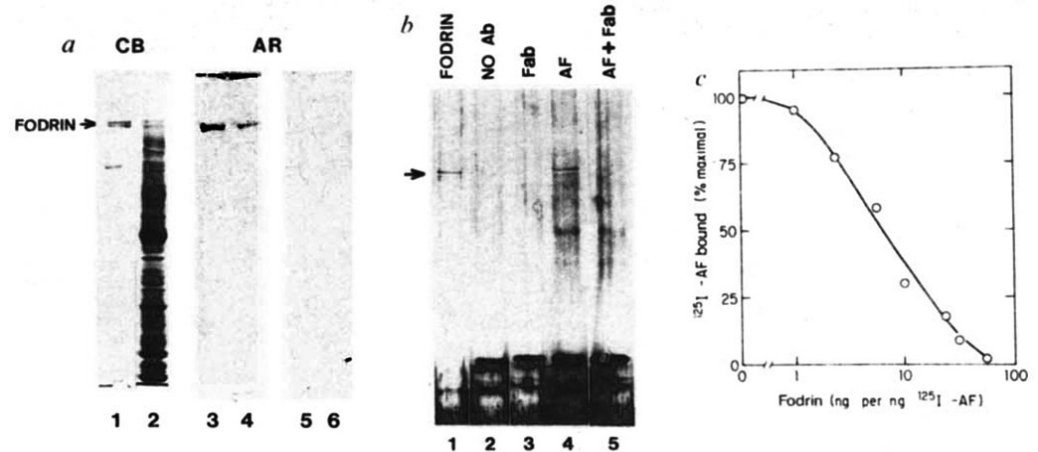
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The erythrocyte cytoskeleton, which consists primarily of a meshwork of spectrin and actin, controls cell shape and the disposition of proteins within the membrane¹⁻³. Proteins similar to spectrin have recently been found in diverse cells and tissues³⁻⁷, and it is possible that they mediate the capping of cell-surface receptors^{8,9}, although this has not been demonstrated directly. In neurones, the spectrin-like protein fodrin lines the cortical cytoplasm and may link actin filaments to the membrane³⁻⁷. Fodrin has been hypothesized to regulate the number of receptor binding sites on neuronal membranes for the putative neurotransmitter L-glutamate^{10,11}. Micromolar calcium concentrations activate the thiol protease calpain I (ref. 12), induce fodrin degradation^{10,11} and more than double the density of glutamate binding sites^{10,13}; these effects are all blocked by thiol protease inhibitors. We have now used specific antibodies to examine further the role of fodrin proteolysis in regulating glutamate receptors. We report that fodrin antibodies block the fodrin degradation and increase in glutamate binding normally induced by calcium, and so provide direct evidence for control of membrane receptors by a non-erythroid spectrin.

The specificity of the anti-fodrin preparation was analysed by the binding of ¹²⁵I-labelled anti-fodrin to electrophoretically separable rat brain membrane polypeptides. Fodrin has previously been identified in synaptosomal plasma membranes (SPMs) as a doublet of relative molecular mass ~240,000 (ref. 11). Anti-fodrin bound to this doublet (Fig. 1a, lanes 2, 4) and to purified fodrin (lanes 1, 3), but did not recognize other SPM polypeptides (lane 4). As well as reacting with denatured fodrin, ¹²⁵I-anti-fodrin bound to the native molecule. When preabsorbed with purified native fodrin, ¹²⁵I-anti-fodrin no longer bound to the fodrin in polyacrylamide gels (Fig. 1a, lanes 5, 6). Furthermore, ¹²⁵I-anti-fodrin bound to hippocampal SPMs, and the binding was prevented by purified fodrin (Fig. 1c). Half-maximal inhibition of the binding of 100 ng of ¹²⁵I-anti-fodrin occurred with 500 ng fodrin, with complete inhibition at 5 μ g fodrin. Monovalent Fab fragments prepared from anti-fodrin IgG retained the capacity to bind to fodrin. The Fab fragments did not, by themselves, precipitate purified fodrin (Fig. 1b, lane

Fig. 1 Specificity of anti-fodrin binding to rat brain membranes. *a*, Binding of 125 I-anti-fodrin to hippocampal synaptosomal plasma membrane polypeptides separated on SDS-polyacrylamide gels. Coomassie blue staining (CB) and autoradiography (AR) of polyacrylamide gel overlaid with 125 I-anti-fodrin²¹. Lanes 1, 3 purified brain fodrin; lanes 2, 4, hippocampal SPMs; lanes 5, 6, purified fodrin (5) or SPMs (6) with 125 I-anti-fodrin preabsorbed with purified fodrin. *b*, Immunoprecipitation of purified fodrin. Lane



1, purified brain fodrin; lane 2, fodrin, no antibody; lane 3, fodrin with 1 µg anti-fodrin Fab; lane 4, fodrin with 0.2 µg anti-fodrin; lane 5, fodrin with 1 µg anti-fodrin Fab and 0.2 µg anti-fodrin. *c*, Binding of 125 I-anti-fodrin (AF) to hippocampal SPMs following preabsorption with various amounts of purified brain fodrin.

Methods: Rat hippocampal synaptosomal plasma membranes, purified fodrin and antibodies to fodrin were prepared as described previously¹¹. Anti-fodrin was purified from rabbit immune serum by affinity chromatography on a column of fodrin coupled to Sepharose 4B (Pharmacia)⁴ and was radioiodinated by the lactoperoxidase method as described previously¹¹. Fodrin and SPMs were run on 6% polyacrylamide slab gels¹⁰ and the gels were overlaid either with 125 I-anti-fodrin (3 µg protein) or with 125 I-anti-fodrin that has been incubated at 22 °C for 30 min with 100 µg purified fodrin (preabsorbed). The gels were washed, stained with 0.1% Coomassie brilliant blue R, destained in 7% (v/v) acetic acid, dried and exposed for 4 days on Kodak XAR-5 film.

For immunoprecipitation, purified rat brain fodrin (p.1 µg) was incubated at 4 °C overnight with the indicated antibodies in 0.2 ml of 50 mM Tris-HCl (pH 7.4), 1 mg ml⁻¹ bovine serum albumin. Fodrin-antibody complexes were recovered by centrifugation (20,000 g for 20 min) and the pellets were washed once with Tris-HCl buffer. The final pellets were suspended in electrophoresis sample buffer and run on 3–10% gradient polyacrylamide gels^{10,11}. Gels were stained with silver²⁰. Monovalent anti-fodrin Fab fragments were prepared by papain digestion²² of anti-fodrin IgG that had been collected by protein A-Sepharose chromatography (Pharmacia, column run according to manufacturer's method). The digest was run over a protein A-Sepharose column to remove undigested IgG, and the Fab fragments in the flowthrough were purified on a fodrin-Sepharose column¹¹ and dialysed against 50 mM Tris-HCl (pH 7.4).

125 I-Anti-fodrin binding to hippocampal SPMs was determined by centrifugation as follows. In 1.5 ml polypropylene centrifuge tubes, 125 I-anti-fodrin (40 ng, 5×10^5 d.p.m.) and various amounts of purified fodrin, determined by protein assay²³, were incubated in 0.05 ml of 50 mM Tris-HCl (pH 7.4) containing 100 µg bovine serum albumin for 30 min at 30 °C. To each tube, 0.45 ml hippocampal SPMs (5 µg protein) in 50 mM Tris-HCl were added, and incubations were continued for an additional 30 min at 30 °C. Membranes were centrifuged at 40,000 g for 20 min, and each pellet was superficially rinsed with 1 ml ice-cold Tris-HCl. Radioactivity retained in the tubes was measured in a gamma-counter (Beckman). Nonspecific retention of 125 I-anti-fodrin was determined by performing incubations in the absence of SPM tissue, and was subtracted from values obtained with tissue present. Specific 125 I-anti-fodrin binding typically represented 80% of the total radioactivity retained. Each data point is the mean from four separate experiments which differed by <20%.

3), but completely prevented the precipitation of fodrin by bivalent anti-fodrin (lanes 4, 5).

To investigate whether calcium-activated fodrin proteolysis increases the number of glutamate binding sites, we tested the effect of anti-fodrin on the calcium stimulation of glutamate binding. Cerebral cortical SPMs were treated with various amounts of anti-fodrin for 30 min before the addition of either EGTA (basal binding) or calcium (basal plus calcium-stimulated binding). In the absence of anti-fodrin, calcium caused an increase in sodium-independent 3 H-L-glutamate binding, with maximal enhancement (200%) occurring at 1 mM calcium (Fig. 2). This increase is due to an elevated B_{max} , with no apparent change in K_d , and probably results from rapid uncovering of binding sites previously hidden in the membranes^{10,14}. Anti-fodrin caused a dose-dependent blockade of the calcium stimulation of glutamate binding (Fig. 2a). A concentration of 150 ng anti-fodrin per µg SPM protein gave complete inhibition, with half-maximal blockade at 60 ng per µg protein. With anti-fodrin 300 ng per µg protein, calcium actually caused a 20% reduction of glutamate binding. Anti-fodrin concentrations below 150 ng per µg protein did not alter basal binding, while slight (~20%) inhibition was detected at higher concentrations. Identical results were obtained with hippocampal SPMs. Monovalent anti-fodrin Fab fragments were equally effective in preventing the calcium stimulation of glutamate binding, 150 ng per µg protein providing >90% inhibition (Fig. 2b). The blockade by anti-fodrin is thus not caused by fodrin cross-linking, as the Fab fragments did not induce fodrin precipitation (Fig. 1b).

We next investigated the mechanism by which anti-fodrin blocked the calcium regulation of glutamate receptor binding.

Anti-fodrin could inhibit the calcium effect by perturbing a fodrin-glutamate binding site interaction, by chelating free calcium, by directly modifying glutamate binding to its receptor, or by nonspecific disturbance of the membrane. First, the ability of anti-fodrin to block the calcium stimulation of glutamate binding was compared with its ability to bind to membrane-associated fodrin. Anti-fodrin was equipotent in blocking 125 I-anti-fodrin binding to hippocampal SPMs and in preventing the calcium stimulation of glutamate binding (Fig. 2a). Second, the anti-fodrin inhibition did not result from chelation of free calcium, as the antibody was as effective at 1 mM calcium as at 0.1 mM (Fig. 2b). Third, an IgG fraction purified from preimmune rabbit serum was without effect on the calcium stimulation of glutamate binding (Fig. 2b), although it reduced basal binding to a similar extent to anti-fodrin. Additionally, anti-fodrin did not prevent all ionic interactions with glutamate binding, as the inhibition of binding caused by millimolar sodium concentrations¹³ was not altered by anti-fodrin. Furthermore, the blockade of 125 I-anti-fodrin binding and of calcium stimulation of glutamate binding persisted in membranes treated with anti-fodrin and then washed free of unbound antibody. Last, anti-fodrin did not block the calcium stimulation of glutamate binding when added after the calcium. Taken together, these results indicate that anti-fodrin prevents the calcium enhancement of glutamate receptor binding by blocking a fodrin-glutamate binding site interaction.

We have proposed previously that calpain I-induced fodrin proteolysis underlies the calcium regulation of glutamate receptor binding^{10,11}. We therefore tested the effect of anti-fodrin on calcium-induced fodrin degradation. Before incubation with

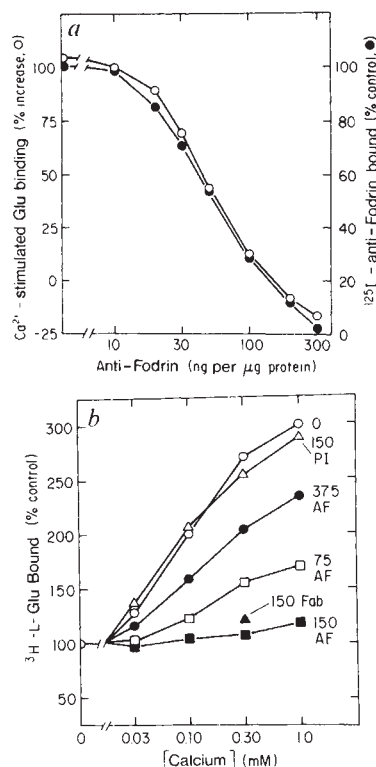


Fig. 2 Effects of anti-fodrin on calcium stimulation of ^3H -glutamate binding and on ^{125}I -anti-fodrin binding. *a*, Dose-responses for anti-fodrin; *b*, anti-fodrin blockade of glutamate binding stimulated by various calcium concentrations. Calcium-stimulated glutamate binding in the presence of antibodies is represented as the percentage of glutamate bound compared with parallel incubations in the presence of EGTA. Each data point represents the mean from four to six experiments. In *b*, the concentrations of anti-fodrin (AF), preimmune IgG (PI) or monovalent anti-fodrin (Fab) are indicated as ng per μg SPM protein.

Methods: Rat cerebral cortical SPMs were suspended in 50 mM Tris-HCl (pH 7.4 at 30°C) and incubated with the indicated amounts of affinity-purified anti-fodrin or preimmune rabbit IgG for 30 min at 30°C, then assayed for either ^{125}I -anti-fodrin binding (*a*, ●) or ^3H -L-glutamate binding (*a*, ○, and *b*). The IgG fraction from preimmune rabbit serum was purified on a column of protein A-Sepharose; the IgG fraction was eluted with 0.2 M glycine-HCl (pH 2.4) and immediately neutralized with 2 M Tris base. All antibodies were dialysed against 50 mM Tris-HCl (pH 7.4) before use. ^{125}I -anti-fodrin binding was determined as described in Fig. 1 legend. Sodium-independent ^3H -L-glutamate binding was measured by a filtration method as described previously^{10,13}. Cortical SPMs were incubated with anti-fodrin or preimmune IgG for 30 min at 30°C, then with 2 mM EGTA (basal-binding) or 0.1 mM calcium (*a*) or various calcium concentrations (*b*) for 15 min. ^3H -L-glutamate (50 Ci mmol⁻¹, ICN) was added to 100 nM and incubations were continued for 15 min.

calcium, hippocampal SPMs were incubated with a concentration of anti-fodrin (300 ng per μg protein) which completely blocks calcium stimulation of glutamate binding. Whereas calcium induced a 22% decrease in fodrin content in normal assay conditions, it has no effect in the presence of anti-fodrin (Table 1). Anti-fodrin did not affect fodrin levels in the absence of calcium. Monovalent anti-fodrin Fab fragments (200 ng per μg protein) also prevented the calcium-induced decline in fodrin content. The antibody probably prevented fodrin degradation by its interaction with fodrin rather than with calpain I, as it did not block the activity of purified calpain I measured against a casein substrate. The strong correlation between anti-fodrin blockade of ^{125}I -anti-fodrin binding and of the calcium stimulation of glutamate binding (Fig. 2*a*) also argues for a direct interaction between fodrin and anti-fodrin, as does the specificity of the antibody for fodrin among all SPM proteins (Fig. 1).

These results implicate the neuronal spectrin-like protein fodrin in the regulation of glutamate receptor binding sites. Because the binding sites are at least partly proteinaceous¹⁵, fodrin can, in a manner similar to erythrocyte spectrin^{1,2}, exert control over plasma membrane proteins. In turn, the levels of fodrin, and perhaps its organization, in the cortical cytoplasm are controlled by calpain I. Activation of this thiol protease by micromolar calcium concentrations leads to a rapid, relatively selective degradation of fodrin^{10,11} and to increased glutamate receptor binding¹⁰. Anti-fodrin, like thiol protease inhibitors^{10,14}, prevents both the glutamate binding increase and the fodrin degradation, but, unlike thiol protease inhibitors, does so without blocking calpain I activity *per se*. This implies a causal role for fodrin proteolysis in regulating the number of glutamate receptor binding sites, and indicates that calpain I does not directly modify these binding sites, but rather alters cytoskeletal control over them. This mechanism for rapid modification of the density of glutamate binding sites may be of considerable functional significance. Pharmacological, biochemical and physiological studies indicate that, at least in the rat hippocampus, ^3H -glutamate may label synaptic neurotransmitter receptors^{16,17}. Indeed, repetitive stimulation of hippocampal Schaffer collateral synapses produces long-term increases both in synaptic efficacy and in the density of glutamate binding sites¹⁸, while learning is accompanied by an increased number of glutamate binding sites in rabbit hippocampal membranes¹⁹.

Table 1 Effect of anti-fodrin on calcium-stimulated fodrin degradation

Condition	Fodrin remaining (% control)	n
No antibodies	78 ± 1*	9
Antifodrin, 300 ng μg^{-1}	101 ± 3	4
Antifodrin Fab, 200 ng μg^{-1}	103 ± 3	5

Hippocampal SPMs (5 μg protein per reaction) were incubated in 50 mM Tris-HCl or in Tris-HCl containing anti-fodrin or anti-fodrin Fab for 30 min at 30°C. Following addition of EGTA (2 mM) or CaCl₂ (0.25 mM), reactions were continued for 30 min at 30°C. They were stopped by the addition of electrophoresis sample buffer^{10,11} and samples were run on 6% slab gels. Polypeptides were visualized by the ultrasensitive silver method of Morrissey²⁰. Fodrin levels were quantified by densitometry^{10,11}. Fodrin remaining after treatment with calcium is taken as the percentage of fodrin content in the presence of calcium compared with its content in the presence of EGTA (control). Each value represents the mean ± s.e.m. * Significantly different from control, $P < 0.005$.

Regulation of glutamate receptors by fodrin could be a useful model system for investigating cytoskeletal-membrane interactions in neurons. Several questions about this interaction remain unresolved. How does fodrin degradation result in cytoskeletal and subsequent membrane protein reorganization? Are other membrane proteins, in particular neurotransmitter receptors, controlled by fodrin? Does fodrin have additional spectrin-like functions, such as regulation of neuronal shape? The finding that brain fodrin can control plasma membrane proteins, as does its erythrocyte counterpart, suggests that the variety of non-erythroid and erythroid spectrins serve similar functional roles.

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Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues

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Epidermal growth factor (EGF), a protein comprising 53 amino acids, is derived from a precursor of 1,217 amino acids that includes at least seven EGF-like sequences^{1,2}. EGF has diverse biological activities (see refs 3-6): it is a potent mitogen for many tissue culture cells, inhibits gastric acid secretion from the intestinal mucosa and promotes healing of the corneal epithelium. EGF given to fetal animals accelerates several developmental processes including palate formation, incisor eruption, eyelid opening and lung maturation. However, the physiological roles of EGF *in vivo* are unknown. The presence of high-affinity receptors in many fetal⁷ and adult tissues⁴ suggests that EGF is involved in normal cellular functions. Immunocytochemical studies have revealed the presence of EGF in mouse⁸ and human submaxillary glands^{9,10}, rat brain¹¹ and human intestine^{9,10}. The low levels of EGF in extracts from many tissues^{12,13} may reflect sequestration rather than synthesis of the polypeptide. We show here that several mouse tissues contain preproEGF mRNA and that it is synthesized mainly in the distal tubules of the kidney. PreproEGF does not seem to be processed to EGF or other peptides in this tissue.

The amount of preproEGF mRNA in different mouse tissues was determined by hybridization of a preproEGF cDNA probe to total RNA (Fig. 1A, B). With the exception of liver, eye and L cells, each sample tested contained this mRNA but the relative abundance varied by three orders of magnitude (Table 1). Poly(A)-containing RNA from the negative sources has not been assessed, therefore the presence of extremely low levels of preproEGF mRNA cannot be ruled out. The data (Table 1) suggest that the EGF found in liver is due to efficient sequestration¹⁴. However, the brain, pancreas, mammary gland and kidney are probably responsible for the EGF found in cerebrospinal fluid¹⁵, pancreatic secretions¹⁶, milk¹⁷ and urine¹⁸, respectively. It is difficult to exclude cross-hybridization of the cDNA probe to related sequences in another mRNA, particularly in instances of low abundance. In the pancreas, though, we observe that a protein the size of EGF is immunoprecipitated from labelled organ cultures (data not shown).

As preproEGF mRNA is present in almost all tissues tested and the serum concentration of EGF is low (1-2 ng ml⁻¹) in both mice and humans⁴, it is difficult to assess the relative

Table 1 Relative abundances of preproEGF mRNA and EGF protein in various mouse tissues

Tissue	Relative preproEGF mRNA abundance	Relative EGF abundance ¹³
Submaxillary gland (male)	2 (1/200)	2,000
Kidney (male, female)	1 (1/400)	1
Submaxillary gland (female)	0.2 (1/2,000)	140
Mammary gland (lactating)	0.01 (1/40,000)	—
Pancreas	0.008 (1/50,000)	0.43
Small intestine (duodenum)	0.004 (1/100,000)	0.33
Pituitary	0.003 (1/150,000)	<0.02
Lung	0.002 (1/200,000)	<0.02
Spleen	0.002 (1/200,000)	—
Brain	0.002 (1/200,000)	<0.02
Ovary	0.002 (1/200,000)	—
Liver	(<1/200,000)	0.33
Eye	(<1/200,000)	—
L cell	(<1/200,000)	—

The relative preproEGF mRNA levels were determined by averaging laser densitometry scans of three dot-blot matrices (for example, see Fig. 1B). Values were normalized to that of the kidney and the numbers in parentheses are the predicted frequency of preproEGF mRNA in poly(A)-containing RNA isolated from the tissues of 8-week-old BALB/c male mice, unless otherwise indicated. Analysis of a cDNA library prepared from male submaxillary RNA indicated that at least 0.5% of the colonies hybridized to a probe encoding the 3'-untranslated region of preproEGF mRNA (J.S. and G.I.B., unpublished). The male submaxillary gland contains ~1 µg of EGF per mg of tissue (wet weight); no values for the EGF content of lactating mammary gland, ovary, spleen, eye or L cells are available. The amount of EGF found in each tissue has been normalized to that determined for the kidney.

contribution of each source to serum EGF. The extensive tissue distribution of EGF receptors and preproEGF mRNA, however, suggests that EGF (or related peptides derived from the precursor) affects either neighbouring cells or those cells that synthesize the protein. These effects may involve, for example, the maintenance of various ductal systems (biliary, gastrointestinal, oesophageal, urogenital) and the response of tissues to injury^{4,19}.

Unexpectedly, the level of preproEGF mRNA in the kidney is only twofold less than that detected in the male mouse submaxillary gland, which contains ~2,000 times more EGF than the kidney (Table 1). As the mRNAs in these two tissues are the same size (N4,900 bases) (Fig. 1C), they probably encode identical proteins. Preliminary evidence indicates that the kidney is also a site of preproEGF mRNA synthesis in humans (L.B.R. and G.I.B., unpublished results). The relatively low levels of EGF in the kidney may reflect differences between the processing of the precursor in this tissue and in the submaxillary gland. Indeed, antibodies to mouse submaxillary EGF or to a segment of preproEGF encoding EGF-like peptides 1, 2 and 3 (Fig. 1A) precipitate a protein from labelled kidney slices (Fig. 1D, lanes 1 and 3, respectively) whose relative molecular mass (M_r) (~130,000) is similar to that predicted for (pre)pro-EGF¹. It is unknown whether the signal peptide is cleaved from this protein. The failure of previous studies^{9,10,12,13} to demonstrate significant amounts of EGF by radioimmunoassay or immunocytochemistry in the mouse or human kidney may be explained by differences in the antibodies used, insolubility of the precursor or masking of antigenic determinants. In the male or female submaxillary gland, anti-mouse EGF antibody precipitates a major protein about the size of EGF (M_r 6,045) and one of M_r 46,000 (Fig. 1D, lanes 4 and 7). The pattern observed with this antiserum and anti-preproEGF-II (Fig. 1D, lanes 6 and 9) is more complex than in the kidney and the relationship of these peptides to EGF and to the precursor is not clear. In neither tissue are any of these proteins secreted into the culture medium (data not shown).

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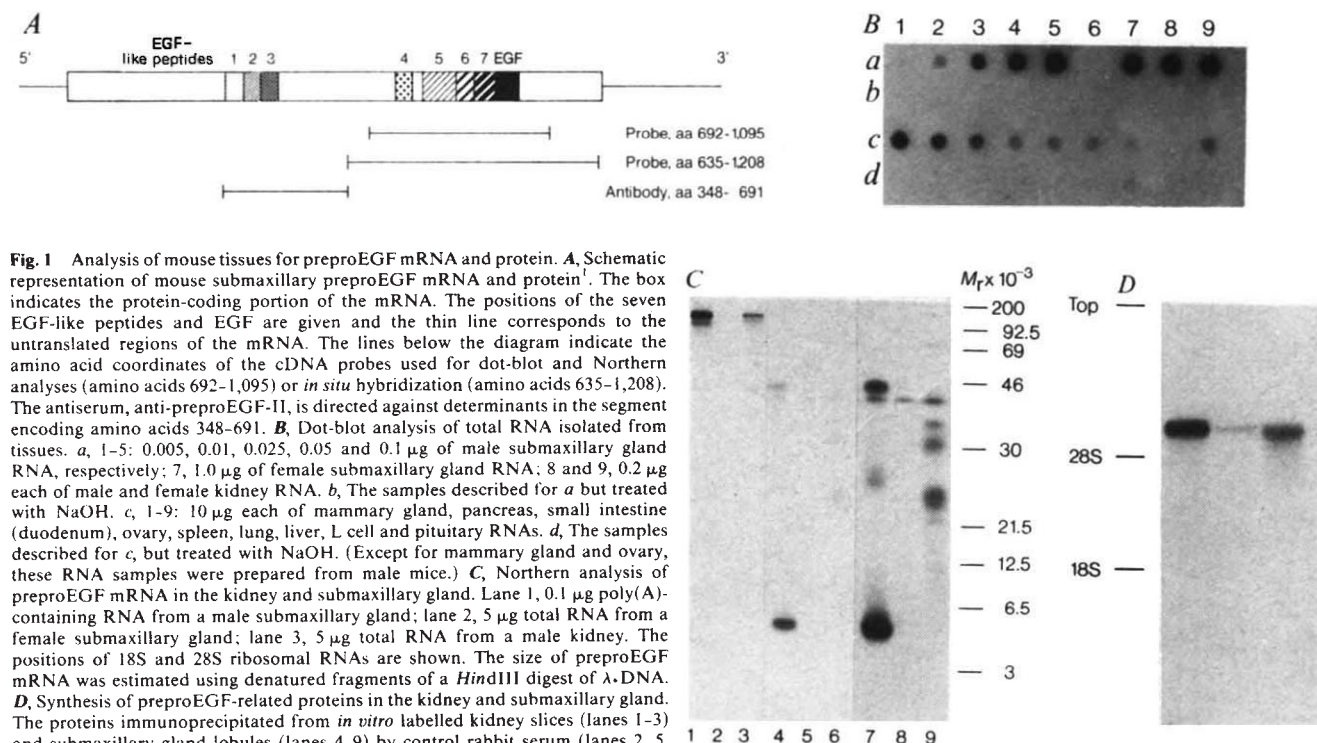


Fig. 1 Analysis of mouse tissues for preproEGF mRNA and protein. **A**, Schematic representation of mouse submaxillary preproEGF mRNA and protein¹. The box indicates the protein-coding portion of the mRNA. The positions of the seven EGF-like peptides and EGF are given and the thin line corresponds to the untranslated regions of the mRNA. The lines below the diagram indicate the amino acid coordinates of the cDNA probes used for dot-blot and Northern analyses (amino acids 692–1,095) or *in situ* hybridization (amino acids 635–1,208). The antiserum, anti-preproEGF-II, is directed against determinants in the segment encoding amino acids 348–691. **B**, Dot-blot analysis of total RNA isolated from tissues. *a*, 1–5: 0.005, 0.01, 0.025, 0.05 and 0.1 μ g of male submaxillary gland RNA, respectively; 7, 1.0 μ g of female submaxillary gland RNA; 8 and 9, 0.2 μ g each of male and female kidney RNA. *b*, The samples described for *a* but treated with NaOH. *c*, 1–9: 10 μ g each of mammary gland, pancreas, small intestine (duodenum), ovary, spleen, lung, liver, L cell and pituitary RNAs. *d*, The samples described for *c*, but treated with NaOH. (Except for mammary gland and ovary, these RNA samples were prepared from male mice.) **C**, Northern analysis of preproEGF mRNA in the kidney and submaxillary gland. Lane 1, 0.1 μ g poly(A)-containing RNA from a male submaxillary gland; lane 2, 5 μ g total RNA from a female submaxillary gland; lane 3, 5 μ g total RNA from a male kidney. The positions of 18S and 28S ribosomal RNAs are shown. The size of preproEGF mRNA was estimated using denatured fragments of a *Hind*III digest of λ -DNA. **D**, Synthesis of preproEGF-related proteins in the kidney and submaxillary gland. The proteins immunoprecipitated from *in vitro* labelled kidney slices (lanes 1–3) and submaxillary gland lobules (lanes 4–9) by control rabbit serum (lanes 2, 5, 8), anti-mouse EGF antibody (Collaborative Research, lot 82-229; lanes 1, 4, 7) and anti-preproEGF-II (prepared as described in ref. 22; lanes 3, 6, 9) were analysed by SDS-polyacrylamide gel electrophoresis²³. Sizes of molecular weight standards are indicated. The gel was exposed for 24 h for lanes 1–6 and for 4 days in the case of lanes 7–9. **Methods:** **B**, RNA was isolated²⁴ from 8-week-old BALB/c mouse tissues and specified amounts were precipitated before dot-blot analysis. The pellets were dissolved in 45 μ l of formamide/formaldehyde buffer²⁵ and heated at 65°C for 10 min. To determine the extent of DNA contaminating the RNA, the RNA was dissolved in 10 μ l of 0.4 M NaOH. The solution was heated at 65°C for 20 min and neutralized with an equal volume of 2 M NH_4OAc . The denatured or hydrolysed RNA was diluted to 200 μ l with 20 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl, 15 mM sodium citrate) and spotted onto a nitrocellulose filter presoaked in 20 $\times$ SSC using a dot-blot apparatus (BRL). The filter was directly dried *in vacuo* at 80°C for 45 min and hybridized with nick-translated ³²P-labelled EGF cDNA insert as described previously²⁶. **C**, RNA was denatured in formamide/formaldehyde²⁵, analysed in a 1% agarose gel, transferred to a nitrocellulose filter and hybridized with nick-translated ³²P-labelled cDNA insert²⁶. **D**, One lobe of a male BALB/c mouse (8 weeks old) submaxillary gland was excised and inflated using a syringe filled with Dulbecco's modified Eagle's medium lacking cysteine and methionine (DMEM, Cys[−], Met[−]) and containing 0.1 mg ml^{−1} soybean trypsin inhibitor (STI). The gland was cut into small sections and incubated for 2.5 h at 37°C in 2 ml of DMEM (Cys[−], Met[−]) containing 0.1 mg ml^{−1} STI. This solution was removed and 2 ml of the same medium containing 50 μ Ci ml^{−1} each of L-[³⁵S]Cys (SJ.232; Amersham) and L-[³⁵S]Met (SJ.204; Amersham) were added. A single kidney was removed, sliced, rinsed in DMEM (Cys[−], Met[−]) and preincubated as above. In both cases, tissue sections were labelled for 4 h, then centrifuged at 4,000 r.p.m. for 10 min at 4°C. The medium was removed and the tissue homogenized in 0.5 ml of 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40 and 0.5% sodium deoxycholate (TNEND). The homogenate was centrifuged for 5 min at 4°C in an Eppendorf microfuge; 5.0 $\times$ 10⁶ acid-precipitable c.p.m. were diluted to 700 μ l with TNEND, then 200 μ l of a 20% solution of Protein A-conjugated Sepharose CL-4B in TNEND, 20% bovine serum albumin were added. The solution was agitated at room temperature for 15 min, then centrifuged in a microfuge for 30 s; 125 μ l of the cleared supernatant were incubated for 2 h at room temperature with 6 μ l of antiserum. The antigen-antibody complex was precipitated, washed and dissociated in 40 μ l of sample buffer as described previously²⁷. The bound antigens were analysed by electrophoresis on a 15% SDS-polyacrylamide gel²³, treated with Enlightening (NEN), dried and autoradiographed.

We next investigated the tissue and cellular sites of synthesis of preproEGF mRNA by *in situ* hybridization (Fig. 2). Comparison of the autoradiograph of a sagittal section of a male mouse with a photograph of the adjacent slice (Fig. 2A, B) indicates that preproEGF mRNA is present in high levels in the cortex of the kidney, in the submaxillary gland and in a region of the mouth possibly coincident with the incisors. (The grains observed in the vertebrae are probably nonspecific as they also occur with several unrelated probes.) The identity of the intensely labelled area within the mouth has not been verified. However, as EGF accelerates incisor eruption in neonatal mice^{3,4}, this region may be involved in the continuous growth of these teeth in rodents.

As expected from the immunocytochemical studies⁸, liquid emulsion autoradiography shows that preproEGF mRNA is produced by the granular convoluted tubules of the submaxillary gland, but the cytoplasmic concentration of grains is greater in male (Fig. 2C) than female cells (Fig. 2D), at least partially accounting for the 10-fold higher levels of EGF observed in the male tissue (Table 1). Lower but equivalent amounts of preproEGF mRNA are found in the striated ducts of the male and female sublingual gland and none was observed in the parotid gland (data not shown).

Low-resolution autoradiography of renal sections (Fig. 2E)

confirmed that preproEGF mRNA is synthesized in the renal cortex and the outer medulla. Consistent with the relative mRNA levels in this tissue (Table 1), no sexual dimorphism is observed and the intensity of labelling is about the same as that found in the male mouse submaxillary gland. The results of liquid emulsion autoradiography localizes synthesis to the cells that constitute the straight and the beginning of the convoluted portion of the distal tubules; preproEGF mRNA levels are higher in the former, and indistinguishable from background in the proximal tubules and collecting ducts (Fig. 2F).

The distal tubules of the kidney are involved in the fine regulation of urine composition²⁰; in particular, Cl[−] is actively transported out of the tubular fluid, aldosterone promotes the reabsorption of Na⁺ and the secretion of K⁺ and H⁺ and in some species including the rat, vasopressin increases the permeability of the distal tubules to water. The function of (pre)proEGF in these kidney cells is unknown. Hydropathic analysis²¹ suggests that preproEGF may be a membrane protein as it contains an internal hydrophobic domain (amino acids 1,039–1,058) adjacent to the EGF moiety (amino acids 977–1,029). If (pre)proEGF is anchored in the basal or luminal surface of the cell membrane, it could be processed to EGF or other biologically active peptides by enzymes present in serum or urine, respectively. It seems unlikely, though, that this is its only

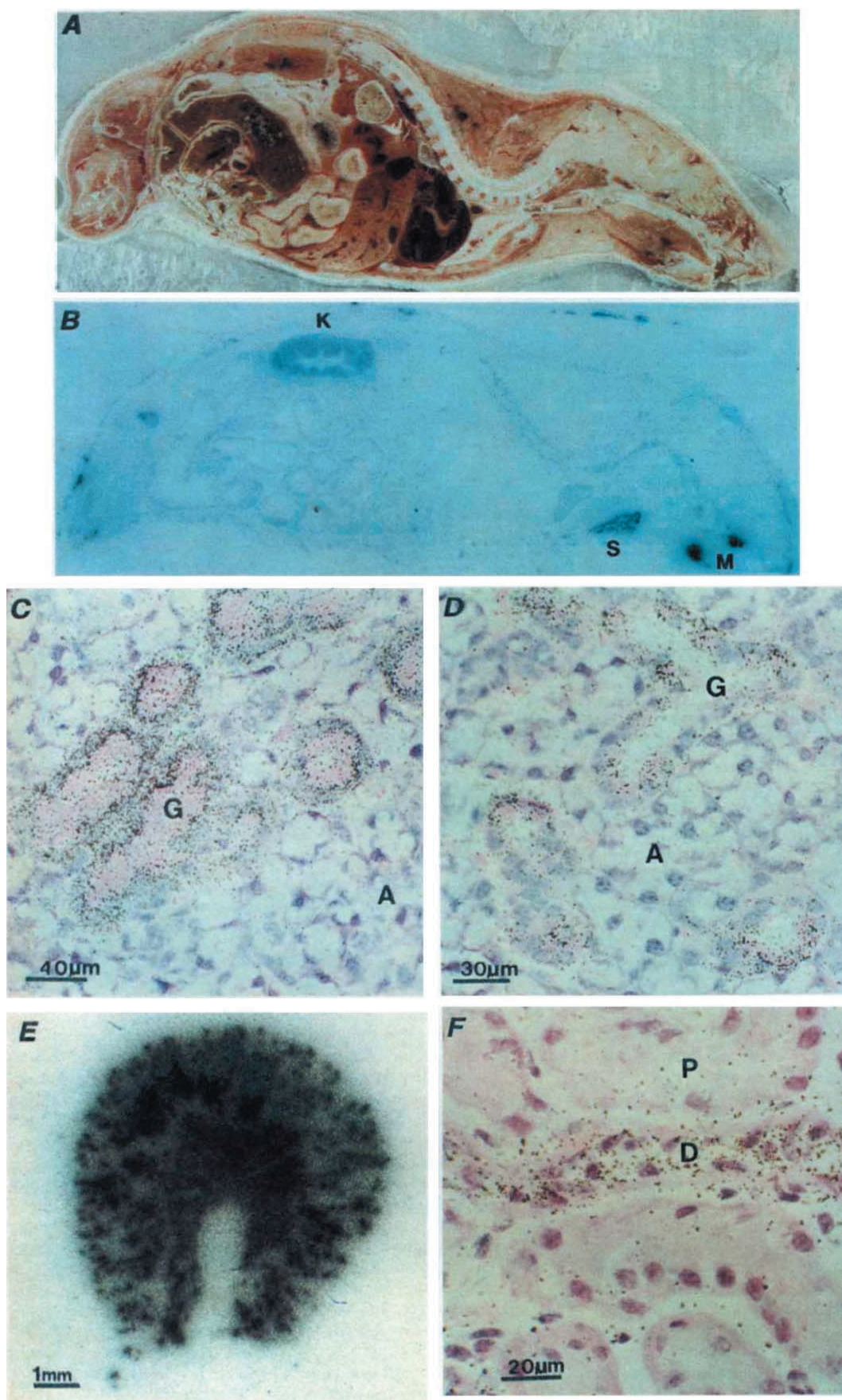


Fig. 2 *In situ* hybridization of preproEGF cDNA to mouse tissues. Sagittal section of an adult male Swiss-Webster mouse. **B–F**, Autoradiographs of sections of a whole mouse (**B**), male submaxillary gland (**C**), female submaxillary gland (**D**), transverse section of a male kidney (**E**) and male kidney cortex (**F**). **B, E** were obtained by direct exposure to X-ray film; **C, D** and **F** were obtained from liquid emulsion autoradiography. **S**, submaxillary gland; **K**, kidney; **M**, mouth region; **G**, granular convoluted tubule cells; **A**, acinar cells; **D**, distal tubule; and **P**, proximal tubule. The distal tubules can be distinguished from the proximal kidney tubules by their wider lumen and thinner walls. **Methods:** Mice were killed by intraperitoneal injection of pentobarbital, frozen in hexane/dry ice and embedded in a 2% carboxymethylcellulose gel. Midline sagittal sections (30–40 µm thick) of the intact mouse were cut at –20 °C using a PMV cryomicrotome (LKB). Isolated slices were adhered to transparent tape and immediately fixed at room temperature in 0.1 M phosphate, pH 7.3 containing 2.5% glutaraldehyde. The sections were rinsed in hybridization solution²⁸, pre-annealed at 40 °C for 2 h, rinsed in ethanol and air-dried. Hybridization with ³²P-labelled preproEGF cDNA (Fig. 1A) was carried out under glass slides for 3 days at 40 °C in a sealed humidified chamber. The sections were then washed briefly in 2×SSC, followed by a final wash for 45 min in 1×SSC at 40 °C. They were rinsed in ethanol, air-dried and directly exposed to X-ray film (Cronex MRF 32; DuPont). For liquid emulsion autoradiography, 4 µm-thick sections of submaxillary glands and kidneys were fixed in sodium phosphate buffer, pH 7.2 containing 4% glutaraldehyde and treated as described previously^{28,29}.

physiological role, and it is tempting to speculate that (pre)pro-EGF functions as a receptor in the cells of the distal tubules, regulating membrane transport events.

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Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist

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About one-third of human breast cancers require hormones for their continued growth¹, and endocrine ablation or anti-hormone therapy can cause regression of these tumours. As a consequence, ovariectomy in premenopausal women or administration of an anti-oestrogen (tamoxifen) in postmenopausal women represent major options for treatment of metastatic breast cancer. Alternatively, chronic administration of agonistic analogues of luteinizing hormone-releasing hormone (LHRH)² causes regression of mammary tumours in experimental animals³⁻⁶, and such treatment has shown promise in a small series of premenopausal women with advanced breast cancer⁷. It has been assumed that these results were achieved by suppressing the pituitary-ovarian axis, as the treatment causes a reduction in circulating levels of gonadal steroids similar to that produced by castration⁶⁻⁸. However, LHRH agonists can exert major effects on tissues other than the pituitary in animals^{9,10} and in the human¹¹⁻¹⁵. Such findings, coupled with reports of LHRH in human breast milk¹⁶ and immunohistochemical evidence for the presence of LHRH-like activity in some human breast tumours¹⁷, prompted us to test whether LHRH agonists could have direct antitumour effects. We now report major direct effects of LHRH and its agonists on the growth of breast tumour cells in culture.

For these studies we used the MCF-7 breast tumour cell line, the growth of which is responsive to sex steroids¹⁸⁻²⁰. In preliminary studies, addition of the LHRH agonist D-Ser-(Bu)¹-LHRH(1-9)-ethylamide (HOE 766; Hoechst) to MCF-7 cells every 2 or 3 days during culture inhibited cell growth, but this effect was not major and was inconsistent. However, when the LHRH agonist was added daily to the MCF-7 cells it caused major consistent suppression of cell growth in a dose-related manner (Fig. 1a). Culture of cells in the presence of concentrations of LHRH agonist in excess of 10⁻⁹ M resulted in a net decrease in cell numbers over the 4-day period of the experiment. These effects were highly reproducible (Table 1). It was also possible to elicit inhibitory effects on cell growth using native LHRH, although much higher concentrations were required than with the LHRH agonist (Fig. 1b). The major inhibitory effects of LHRH agonist on cell growth were blocked completely by addition of the LHRH antagonist [N-Ac-D-Nal(2)¹, D-pCl-Phe², D-Trp³, D-Har(Et)⁶, D-Ala¹⁰]LHRH, the combination of this peptide and the agonist having no greater effect than the antagonist alone (Fig. 1c); the latter caused only minor but statistically significant suppression of cell growth.

The fact that the LHRH antagonist blocks the inhibitory effects of the LHRH agonist on the tumour cells suggests the involvement of specific receptors. Using whole cell preparations of the MCF-7 line, we were able to demonstrate specific binding of ¹²⁵I-labelled LHRH agonist (Fig. 2), for which LHRH showed minor but significant competition whilst thyrotropin releasing hormone (TRH) had no effect. The concentrations of LHRH agonist required for displacement (10⁻⁶-10⁻⁴ M) indicate that the binding sites are of apparently low affinity when compared with extrapituitary binding sites for LHRH agonists in the rat^{9,21}. However, the present observations are similar to those in other human extrapituitary tissues (ovary, testis and placenta) which also possess low-affinity binding sites for LHRH agonists^{13,22-24}. Moreover, in these tissues^{11,15,24-26}, as in the MCF-7 cells studied here, concentrations of LHRH agonist which are insufficient to displace the binding of radiolabelled tracer under receptor-assay conditions (10⁻⁹ M) are capable of eliciting clear-cut biological

Table 1 Reproducibility of the inhibitory effects of LHRH agonist on cell numbers during culture of the MCF-7 line

Concentration of added LHRH agonist (M)	No. of experiments	Day of culture			
		1	2	3	4
0	(7)	1.35 ± 0.19	1.93 ± 0.39	2.49 ± 0.36	2.89 ± 0.39
10 ⁻⁹	(5)	1.14 ± 0.06*	1.07 ± 0.13§	0.77 ± 0.11§	0.64 ± 0.15§
10 ⁻⁷	(3)	0.96 ± 0.05‡	0.90 ± 0.14‡	0.58 ± 0.12§	0.39 ± 0.10§
10 ⁻⁶	(2)	0.89 ± 0.10†	0.85 ± 0.04‡	0.51 ± 0.01§	0.15 ± 0.07§

Values are the mean ± s.d. and refer to the ratio of cell numbers on the day of collection to that (0.5 × 10⁶) plated initially on day 0. Experimental conditions and statistical analysis were as described for Fig. 1.

* P < 0.05; † P < 0.02; ‡ P < 0.01; § P < 0.001; in comparison with respective control values (Student's *t*-test).

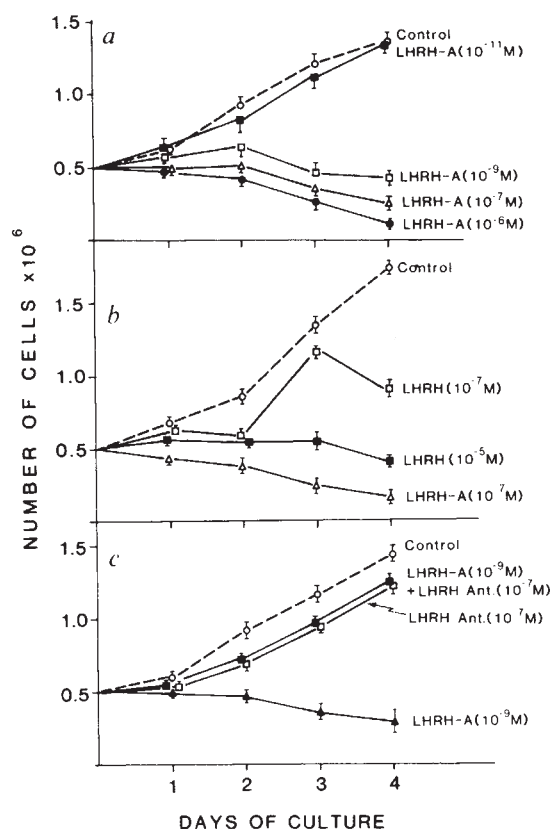
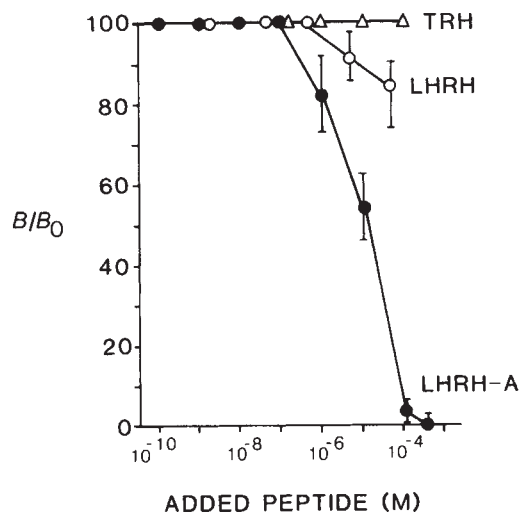


Fig. 1 Inhibition of the growth of MCF-7 breast tumour cells by LHRH and its agonists and blockade of inhibition by an LHRH antagonist. *a*, Dose-response relationship for the LHRH agonist (LHRH-A). *b*, Comparison of LHRH and the LHRH agonist. *c*, Effect of the agonist or an antagonist of LHRH (LHRH Ant.), either alone or in combination. In *a*, all doses of LHRH agonist, other than 10^{-11} M, caused significant ($P < 0.001$) overall suppression of cell growth compared with the control, in *b* LHRH agonist and both doses of LHRH caused significant ($P < 0.001$) suppression of growth, whilst in *c*, LHRH antagonist caused minor but significant ($P < 0.001$) suppression of growth and the two hormones together had no greater effect ($P > 0.1$) than the LHRH antagonist on its own.

Methods: MCF-7 cells were provided by Michigan Cancer Foundation and were used at their 183rd passage. The cells were grown at 37°C in Dulbecco's minimal essential medium containing 10% heat-inactivated fetal calf serum and 16 mM sodium bicarbonate in 175-cm^3 flasks under a humidified atmosphere of 5% CO_2 : 95% air. Cells growing in log phase were then collected (= day 0) and 0.5×10^6 cells in 4 ml culture medium were plated out in 60 mm Petri dishes. The various hormones were added after being dissolved in culture media at the appropriate concentration and sterilized by filtration. All media were replaced daily. Petri dishes were incubated at 37°C as described above for the periods indicated. Cells were collected after removal of culture media by washing with 0.02% EDTA in 1 ml phosphate-buffered saline (PBS) followed by incubation with 0.1% trypsin in 4 ml PBS for 5 min at 37°C . Cells were then counted in a haemocytometer. The values shown represent the mean and range of triplicate cultures at each time point and hormone concentration. The experiments illustrated are representative of at least two similar experiments (see also Table 1). Results were analysed using two-factor analysis of variance to indicate whether there was an overall significant effect of a particular hormone treatment. Where such differences were observed, further comparison of results for individual days was done using Student's *t*-test.

Fig. 2 Displacement of the binding of ^{125}I -labelled LHRH agonist to isolated MCF-7 breast tumour cells by unlabelled LHRH agonist, native LHRH and TRH. All doses of LHRH agonist above 10^{-7} M and the two doses of LHRH above 10^{-6} M suppressed binding significantly ($P < 0.05$ to $P < 0.001$).

Methods: Cells were grown as described in Fig. 1 and collected from flasks by flushing medium in and out using a Pasteur pipette. The cells were then washed in M199 (Flow) containing Hank's salts, 20 mM HEPES and bovine serum albumin (BSA; fraction V, Sigma; 0.25 mg ml^{-1}), precipitated by centrifugation at 4°C for 5 min at 250g and then resuspended in the same medium. Aliquots of 1.2×10^6 cells were then incubated in 63×11 mm polystyrene tubes together with 200,000 c.p.m. ^{125}I -labelled LHRH agonist²² and unlabelled hormones at the indicated concentrations in a final volume of 0.3 ml. Cells were incubated for 10 min at 21°C and then placed on ice for 10 min. After addition of 2 ml ice-cold medium the cells were precipitated by centrifugation at 4°C for 10 min at 1,000g, and radioactivity in the precipitate measured. The total tracer bound to cells was 18,000 c.p.m. and of this 10,000 c.p.m. was displaced by 5×10^{-4} M unlabelled LHRH agonist; the remaining 8,000 c.p.m. was considered to represent non-specific or non-displaceable binding and was subtracted from all values for construction of the figure. Values show the mean and range of quadruplicate incubations.



effects. This paradox is unexplained but it may be simply a reflection of the different time periods and temperatures used for assessment of the receptor-binding potency of LHRH agonists and that used for assessment of the biological potency. Alternatively it is possible that high-affinity binding sites for LHRH agonist are present in MCF-7 cells and these other tissues, but that they represent a small proportion of the total binding sites present. It is also noteworthy that, in the present studies, maximum binding of radiolabelled LHRH agonist was obtained after only 10 min incubation at 21°C . Longer incubation (20–30 min) resulted in a marked decrease in binding (data not shown), presumably as a result of proteolytic degradation. Again, this is similar to findings for binding of LHRH agonist to the human ovary, testis and placenta^{13,22,24}. This presumed

proteolytic activity associated with MCF-7 cells would also explain our inability to show consistent inhibitory effects of the LHRH agonist on cell growth unless the media were replenished with fresh LHRH agonist every 24 h.

To our knowledge, the present data represent the first unequivocal demonstration that LHRH is capable of exerting major direct effects on the growth of human breast cancer cells. The possibility that these effects represent an anti-steroidal action, as has been reported for LHRH agonists in other tissues^{27,28}, cannot be excluded at this stage, as the growth of the MCF-7 cell line may be stimulated by small amounts (10^{-11} M) of oestradiol²⁹ such as are present in the fetal calf serum included in these cultures.

At present, trials with LHRH agonists are being conducted

in premenopausal women as a form of medical ovariectomy^{30,31}, but if direct effects on breast tumour cells are possible there is no reason why application of this therapy should be restricted to premenopausal patients. Indeed, treatment with LHRH agonist can produce objective responses in some postmenopausal women with breast cancer and inactive ovaries³², results which are consistent with direct antitumour effects of the LHRH agonist. However, as the present findings suggest that the LHRH agonist had to be replenished daily to be effective in inhibiting tumour cell growth, then conventionally used doses of LHRH agonists and their method of administration to patients are probably insufficient to produce drastic direct antitumour effects. Infusion or implantation techniques would be required and such approaches are currently being developed by pharmaceutical companies^{33,34}.

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Homing receptor-bearing thymocytes, an immunocompetent cortical subpopulation

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Much of the differentiation of murine T cells takes place in the thymus¹⁻⁶, perhaps influenced by the operation of stringent selection mechanisms whose existence has been inferred from the high rate of thymocyte turnover in the absence of extensive emigration⁷⁻¹¹. The origin of those 1% of total thymocytes which leave the thymus and seed the peripheral lymphoid organs is obscure. Recent thymic emigrants are functionally and phenotypically mature¹²⁻¹⁴, and the purported greater maturity of medullary relative to cortical thymocytes is often cited as evidence for the medullary origin of thymic emigrants¹⁵, a suggestion not without its critics^{16,17}. To approach this question, we have now isolated a subpopulation of thymocytes expressing high levels of a receptor that mediates the homing of blood-borne lymphocytes into peripheral lymph nodes. Surprisingly, this population of cells (1-3% of total thymocytes) is both cortical and immunocompetent, containing approximately half of all thymic cytolytic T-lymphocyte precursors. The combination of homing receptor expression and immunocompetence makes this cortical population ideally suited for emigration to peripheral lymphoid organs.

The migration of recirculating lymphocytes from the blood into lymph nodes and Peyer's patches is initiated by their selective adherence to the endothelial cells lining the post-capillary high endothelial venules¹⁸⁻²⁰. One structure on the surface of the murine lymphocyte which is involved in this interaction is a glycoprotein of relative molecular mass 80,000 recognized by the recently described rat monoclonal antibody MEL-14 (ref.

21). Preincubation of lymphocytes with MEL-14 specifically blocks their migration into lymph nodes *in vivo* and prevents their attachment to high endothelial venules in tissue sections from peripheral lymph nodes but not from Peyer's patches²¹. Thus, murine lymphocyte recirculation seems to be directed by the interaction between determinants differentially expressed on peripheral node and Peyer's patch high endothelial venules and receptors for these determinants expressed by lymphocytes. Peripheral B and T cells generally express homing receptors that can interact with both peripheral node and Peyer's patch determinants²¹⁻²³.

Most thymocytes express only low levels of the determinant recognized by MEL-14 and accordingly bind poorly to peripheral node endothelial venules²¹. However, staining of thymic sections with MEL-14 has revealed three populations based on homing receptor expression: a MEL-14 negative to low medullary population, a major MEL-14 low to intermediate cortical population, and a rare population of medium-large cells scattered throughout the cortex which expresses high levels of the MEL-14 determinant¹⁷. We reasoned that the expression of receptors for homing to peripheral lymphoid organs would be associated with a late stage of intrathymic differentiation, an argument supported by the observation that these receptors are not expressed by thymocytes recently derived from the bone marrow, but that they are expressed by most peripheral T cells newly emigrated from the thymus¹⁷. We therefore analysed, in a limiting dilution assay of the thymic cytolytic T lymphocyte (T_c) precursors, the immunocompetence of that population of cortical cells which stains brightly with MEL-14.

Viable thymocytes stained with MEL-14 were sorted by flow cytometry into two populations: those expressing the 2-3% highest levels of the MEL-14 determinant (MEL-14^{hi}) and those expressing the lowest 45-55% levels (MEL-14^{lo}). The fluorescence profile of thymocytes stained with MEL-14 shows a shoulder of brightly stained cells (Fig. 1a) which can be successfully separated from the bulk of thymocytes (Fig. 1c). Forward light scatter profiles also reveal the relatively large size of the MEL-14^{hi} thymocytes obtained, an observation made previously in the analysis of tissue sections¹⁷.

Separated and unseparated BALB/c (H-2^d) thymocytes were cultured in the presence of interleukin-2-containing supernatants with irradiated allogeneic B6 (H-2^b) spleen cells, and assayed 7 days later for T_c-cell activity. Initial experiments

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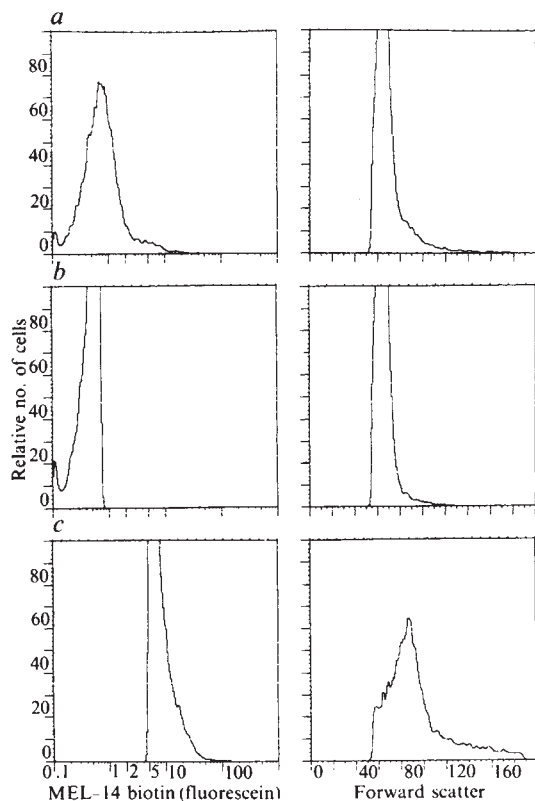


Fig. 1 Forward light scatter (right panels) and fluorescence profiles (left panels) of MEL-14-stained thymocytes. Thymuses from 7-week-old BALB/c females were removed free of parathymic lymph nodes, and 10^8 thymocytes were stained with 2 ml biotin-conjugated MEL-14 at concentration of $200 \mu\text{g ml}^{-1}$, followed by 2 ml fluorescein-conjugated avidin (Vector Laboratories) at a concentration of $100 \mu\text{g ml}^{-1}$. Viable cells were sorted with coincidence circuits in, using a fluorescence-activated cell sorter (modified FACS II, Becton-Dickinson). Fluorescence profiles (fluorescence intensity in arbitrary logarithmic units) and forward light scatter profiles used as a measure of cell size (arbitrary linear units) are from *a*, unseparated thymocytes; *b*, thymocytes sorted for expression of the lowest 50–55% levels of MEL-14; *c*, thymocytes sorted for expression of the highest 2–3% levels of MEL-14. Control thymocytes incubated with biotinylated isotype-matched negative control antibody and fluorescein-avidin showed only background levels of staining. Note the shoulder of MEL-14^{hi} cells in the unseparated population, the efficiency of the separation on the basis of MEL-14 expression, and the larger size of the MEL-14^{hi} relative to MEL-14^{lo} thymocytes.

performed in bulk culture revealed a >10-fold difference between MEL-14^{hi} and MEL-14^{lo} thymocytes in the amount of cytolytic activity per recovered cell, with the MEL-14^{hi} population showing the highest activity. To quantify the number of T_c-cell precursors in unseparated, MEL-14^{hi} and MEL-14^{lo} populations of thymocytes, mixed lymphocyte cultures were carried out in limiting dilution. Figure 2 shows the data from four independent limiting dilution experiments with separated thymocyte responder cells. These independent isolates of MEL-14^{hi} thymocytes are strikingly uniform in their numbers of anti-H-2^b T_c-cell precursors, as are the unseparated thymocytes. The greater variation among the MEL-14^{lo} populations can perhaps be ascribed to a greater variation in the sorting gates between experiments (required to separate an adequate number of these relatively inactive cells for analysis), coupled with the intermediate T_c-cell precursor frequency of thymocytes expressing intermediate levels of the MEL-14 determinant (data not shown). These data illustrate that separation of thymocytes on the basis of high levels of homing receptor expression efficiently enriches for precursor T_c cells: MEL-14^{hi} thymocytes have a 250-fold higher alloreactive T_c-cell precursor frequency than do MEL-14^{lo}

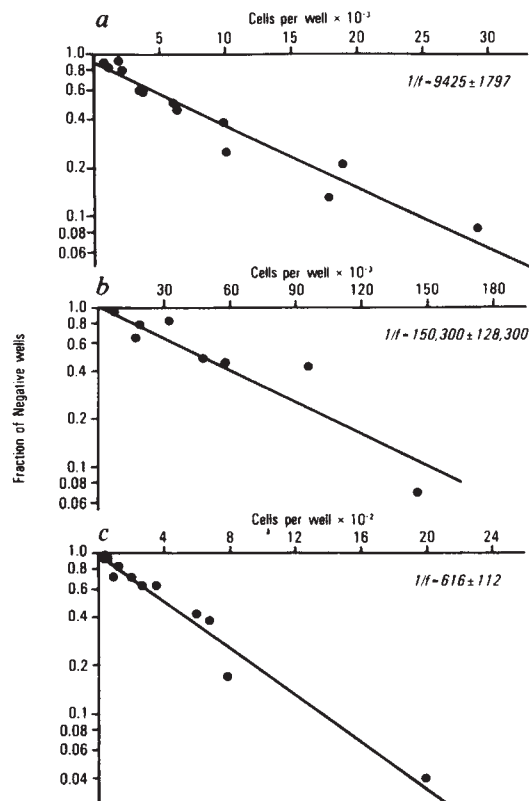


Fig. 2 The MEL-14^{hi} population of thymocytes contains alloreactive T_c-cell precursors. Thirteen to 30 wells each of several serial dilutions of separated and unseparated thymocytes from 6–7-week-old BALB/c (H-2^d) mice were cultured in round-bottomed 96-well plates (Costar) with 10^6 allogeneic B6 (H-2^b) irradiated spleen cells. Cultures contained α -methyl-D-mannoside and 16% supernatants from Con A-activated rat spleen cells. After 7 days, cultures were assayed for T_c-cell activity in a 4-h assay, using as target cells ⁵¹Cr-labelled EL4 (H-2^b) cells (7–9% spontaneous release) or B6 3-day Con A blasts (14–25% spontaneous release). Positive wells were those in which the amount of ⁵¹Cr released exceeded by three standard deviations that released in wells containing stimulators alone. Responding cells were: *a*, unseparated thymocytes; *b*, MEL-14^{lo} thymocytes; *c*, MEL-14^{hi} thymocytes. Also shown are the mean inverse precursor frequencies ($1/f$) $\pm$ s.e.m. from four independent experiments. Precursor frequencies were calculated by weighted mean analysis³⁴ and curves were drawn according to parameters calculated by the least-squares method³⁵. The coefficients of determination for the individual experiments were: *a*, 0.97–1.00 (0.94 for combined experiments); *b*, 0.99–1.00 (0.83 for combined experiments); *c*, 0.98–0.99 (0.97 for combined experiments).

thymocytes. In fact, if MEL-14^{hi} thymocytes comprise 3% of total thymocytes, this population would contain about half the anti-H-2^b precursor T_c cells contained within the entire thymocyte population. This enrichment in cytolytic precursors is also evident among large cells separated by elutriation and secondarily sorted according to MEL-14 expression (data not shown), and is therefore a function, not of size, but of MEL-14 expression. The MEL-14 determinant is clearly expressed on a select population of immunocompetent thymocytes.

The MEL-14^{hi} population of BALB/c thymocytes includes a large number of T_c precursors specific not only for H-2^b, but also for H-2^k, H-2^s and trinitrophenyl (TNP)-modified self H-2^d, with 50–250-fold higher activity in the MEL-14^{hi} than the MEL-14^{lo} population (Table 1). The low number of MEL-14^{hi} cells available prevents the inclusion of a full range of specificity controls in these experiments. However, extensive experiments with unseparated thymocytes indicate that these limiting dilution conditions result in activation mainly of specific T_c-cell precursors (data not shown). With the caveat that MEL-14^{hi} cells may have different activation requirements and that some limiting dilution conditions result in activation of T_c cells that have a

Table 1 T_c-cell precursor frequency of three populations of thymocytes

Responding BALB/c thymocytes	Inverse precursor frequency				
	Anti-B6	Anti-BALB-K	Anti-A-SW	Anti-BALB/c plus TNP	Anti-B10-D2
MEL-14 stained	9,425	5,057	22,720	63,310	42,100
MEL-14 low	150,300	108,800	142,800	852,600	30,540
MEL-14 high	616	661	1,215	16,780	37,690

Separated and unseparated BALB/c thymocytes were cultured in limiting dilution as described in Fig. 2 legend, using as stimulator spleen cells from B6 (H-2^b), BALB-K (H-2^k), A-SW (H-2^s), BALB/c conjugated with TNP³³, and B10-D2 (H-2^d plus minor H). Responder thymocytes used to generate minor H-specific cells were derived from mice injected intraperitoneally 13 days previously with 1.5×10^7 viable B10-D2 spleen cells. ⁵¹Cr-labelled target cells were EL4 (7–9% spontaneous release) or 3-day concanavalin A stimulated (Con A) blasts of the stimulator cell type, spontaneously releasing 11–26% of ⁵¹Cr during the 4-h T_c-cell assay. Anti-B6 frequencies were averaged from four experiments (Fig. 2), anti-B10-D2 from three experiments, and the remaining data calculated by weighted mean analysis of single experiments.

variety of specificities^{24,25}, we can conclude that the diversity of the cytolytic response by MEL-14^{hi} thymocytes is so far indistinguishable from that of unseparated thymocytes or peripheral T cells. The frequencies calculated from these primary cytolytic responses can be contrasted with those obtained from the thymic cytolytic response to minor histocompatibility (minor H) antigens, which requires *in vivo* priming²⁶. The minor H-specific T_c-cell precursor frequency is the same among the separated thymocyte populations, perhaps as a result of this priming. One component of the thymic minor H-specific memory T_c-cell response is due to the activity of mature peripheral T cells which have returned to the thymus²⁶; moreover, T cells that have undergone antigen-specific or lectin-mediated stimulation are known to temporarily lose homing receptor and the MEL-14 determinant^{21,27}. Thus, both thymocytes recently primed to minor H and antigen-activated peripheral T_c cells which re-enter the thymus would be expected to express low levels of the MEL-14 determinant. These data therefore emphasize both the diversity of the MEL-14^{hi} intrathymic T_c-cell response and the lack of contribution to this response by mature peripheral T cells^{26,28}.

The experiments reported here define a subpopulation of thymocytes which expressed high levels of a peripheral lymph node homing receptor, and which includes at least one-quarter to one-half of the alloreactive T_c-cell precursors contained within the entire thymocyte population. The cortical location of MEL-14^{hi} thymocytes has been clearly shown by immunoperoxidase staining of tissue sections¹⁷, and is supported indirectly by evidence such as the medium-large size of many MEL-14^{hi} thymocytes (Fig. 1c and ref. 17). In contrast to their cortical location, other characteristics of MEL-14^{hi} cells, such as cortisone resistance¹⁷ and the levels of expression of surface markers (for example, H-2, Thy-1, L3T4, Lyt-1, Lyt-2, and peanut agglutinin receptors; R.A.R., in preparation), classify MEL-14^{hi} thymocytes as mature cells. Thus, previous studies defining the medulla as the source of immunocompetent thymocytes could have categorized MEL-14^{hi} cortical cells as 'medullary'^{29–32}. The identification of a mature subpopulation of thymocytes scattered throughout the cortex therefore reopens the question of the immediate origin within the thymus of the cells which will emigrate and contribute to the pool of functional peripheral T cells. Because the high levels of expression of the MEL-14 determinant by the T_c-cell precursor-containing cortical population distinguishes these cells and recent thymic emigrants from medullary cells¹⁷, the most 'parsimonious' model suggests that the emigrants are derived from the pool of functional, MEL-14^{hi} cortical thymocytes. Our data do not permit localization either of the remaining T_c-cell precursors or of any immunocompetent, non-cytolytic thymocytes. Perhaps an analysis of cells expressing functions other than cytolytic activity will clarify the role of medullary thymocytes.

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Haemoglobin switching in human embryos: asynchrony of $\zeta \rightarrow \alpha$ and $\epsilon \rightarrow \gamma$ -globin switches in primitive and definitive erythropoietic lineage

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Haemoglobin switching in humans provides a unique model for investigating the mechanisms underlying expression of a developmentally regulated gene family. Numerous studies have focused on the switch from fetal to adult (that is, $\gamma \rightarrow \beta$) globin^{1,2}, but little is known about the embryonic $\rightarrow$ fetal (that is, $\zeta \rightarrow \alpha$ and $\epsilon \rightarrow \gamma$) switches, as well as the transition from 'primitive' yolk sac to 'definitive' liver erythropoiesis^{3–7}. Here we have studied the embryonic $\rightarrow$ fetal haemoglobin switches in yolk sac, liver and circulating blood erythroblasts from 25 embryos and 6 fetuses. Globin synthesis was also evaluated in purified 'primitive' and 'definitive' erythroblasts. Primitive erythroblasts synthesize essentially ζ and ϵ chains at 5 weeks and α - and ϵ -globin with a minor

aliquot of ζ and γ chains at 6–7 weeks, whereas definitive erythroblasts produce α and $\epsilon + \gamma + \beta$ -globin at 6 weeks but only α and $\gamma + \beta$ chains from 8 weeks onward. In both lineages the $\zeta \rightarrow \alpha$ and the $\epsilon \rightarrow \gamma$ switches are asynchronous, the former preceding the latter. Furthermore, ζ - and β -globin synthesis is restricted to primitive and definitive erythroblasts respectively. These findings are discussed in terms of a monoclonal model for haemoglobin switching in early human ontogeny.

In human embryos, ontogenic development of the erythron entails parallel switches of erythropoietic site, erythroblast morphology and haemoglobin (Hb) content^{3–7}. The primitive yolk sac-derived lineage consists of nucleated 'megalo-blasts', which appear 2–3 weeks after conception^{7–9} and are present in circulating blood from 4 to 10–12 weeks^{3,4}. The definitive lineage consists of 'macrocytic' erythroblasts, which arise in the liver at 6–7 weeks and generate enucleated red cells which enter the circulation from about 8 weeks onward^{3,4,7}. In the earliest embryos examined so far (~7 weeks old), haemoglobin Gower 1 ($\zeta_2\epsilon_2$), Gower 2 ($\alpha_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$) account for ~65% of the total haemoglobin content in blood¹⁰; they are then rapidly replaced by fetal haemoglobin (HbF; $\alpha_2\gamma_2$) and some adult haemoglobin (HbA; $\alpha_2\beta_2$), which are virtually the only haemoglobins present in peripheral blood from 10–12 weeks onward^{3–5,10}.

Adequate analysis of the embryonic $\rightarrow$ fetal haemoglobin switch has been virtually precluded so far, for three reasons. (1) Yolk sac, liver and blood from 5–6-week-old embryos have simply not been available for studies of haemoglobin^{5,10}. (2) Globin synthesis in blood, evaluated only in embryos and fetuses at 8–34 weeks, failed to demonstrate production of embryonic chains^{11,12}, hence the timing of the embryonic $\rightarrow$ fetal switch has been grossly extrapolated from haemoglobin content in 7–10-week-old blood⁵. (3) Although primitive and definitive erythroblasts may coexist in liver and blood, no information was available on their differential embryonic haemoglobin content and/or synthesis.

Here we have analysed yolk sac, liver and blood erythroblasts from 25 embryos and 6 fetuses, obtained virtually intact from legal curettage abortions 5–10 weeks after conception. The age of 5–8-week-old embryos was carefully established by morphological staging, according to multiple, precise criteria^{13,14}; 9–10-week-old fetuses were staged on the basis of standard age/crown-rump length (CRL) plots^{14,15}. The integrity of the examined embryos and fetuses gave a dating error of as little as ± 2 days^{13,14}. Yolk sacs were available only from 5-week-old embryos. Erythroblasts from liver and circulating blood were obtained from 5 or 6 weeks to 10 weeks respectively.

At 5 weeks, yolk sacs contain only megaloblasts at all stages of maturation (Fig. 1a), which are simultaneously present in circulating blood, and particularly in intrahepatic sinusoids (Fig. 1b). There was no evidence of either primitive or definitive erythropoiesis in 5-week liver parenchyma. Definitive erythroblasts appear in 6-week-old liver and enter the circulation at 8 weeks⁷. The ratio of primitive to primitive plus definitive erythroblasts in liver is 1.00 at 5 weeks, 0.69 ± 0.15 at 6 weeks, 0.15 ± 0.05 at 7 weeks, and <0.05 from 8 weeks onward (mean $\pm$ s.e.m.). Circulating blood contains primitive erythroblasts at 5–7 weeks (see below) and a mixed population at 8–12 weeks (not shown).

The pattern of globin synthesis was analysed by Triton-urea electrophoresis (TUE)¹⁶ and/or urea-Nonidet P-40 (NP40) isoelectric focusing (IEF)¹⁷ of ³H-labelled globin chains (Fig. 2a, b). In 5-week-old embryos (CRL <10 mm, stage 14–15), erythroblasts from yolk sac, liver and blood show an identical pattern of globin synthesis, that is, predominant synthesis of embryonic ζ - and ϵ -globin ($\zeta/\zeta + \alpha = 0.82 \pm 0.04$; $\epsilon/\epsilon + \gamma = 0.83 \pm 0.05$). In one case, we observed $>95\%$ embryonic globin synthesis in both yolk sac and liver erythroblasts. In 6-week-old embryos (CRL 10–15 mm, stage 16–17), the ζ chain is almost completely replaced by α -globin in liver erythroblasts ($\zeta/\zeta + \alpha = 0.03 \pm 0.02$), whereas the ϵ chain is still synthesized at relatively high levels ($\epsilon/\epsilon + \gamma = 0.31 \pm 0.10$). In four out of six cases,

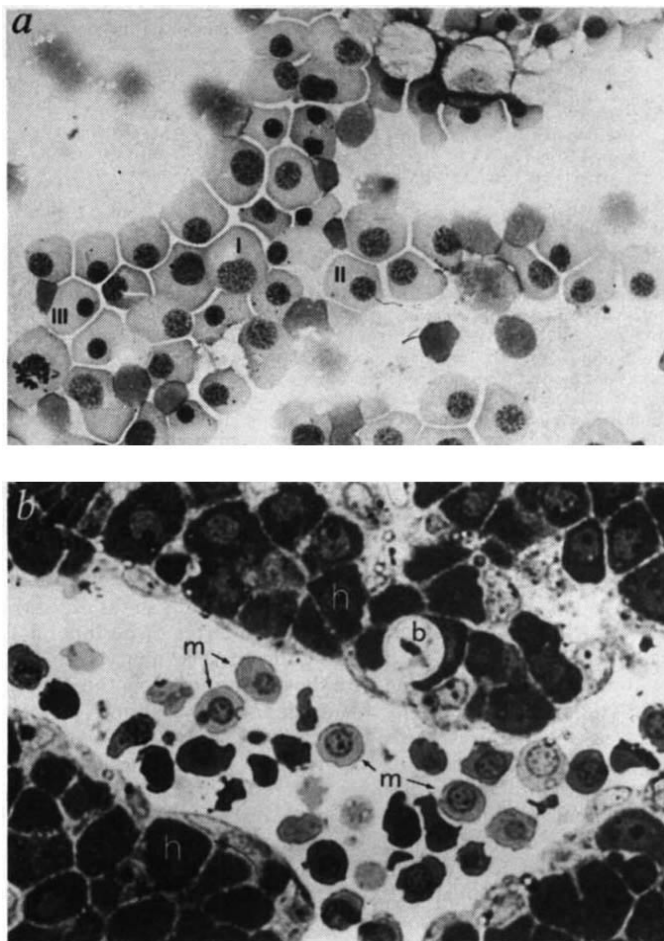


Fig. 1 a, Erythroblasts of the primitive lineage from human yolk sac 5 weeks after conception. Virtually intact embryos, obtained from legal curettage abortions, were maintained in RPMI 1640 medium (Flow) for up to 6 h. A single-cell suspension¹⁹ was cytocentrifuged and stained with eosin-methylene blue (May-Grünwald) solution by standard procedures⁷. The ratio of erythroblasts to total nucleated cells was ~0.60. I, II and III indicate erythroblasts at different stages of maturation, according to ref. 7. b, Section of 6-week-old liver stained with toluidine blue. m, Circulating megaloblasts; b, undifferentiated blast cell; h, hepatocyte.

synthesis of adult β -globin was clearly detectable (~1.5% of non- α -globin). In 7-week-old embryos (CRL 16–20 mm, stage 18–20), liver erythroblasts produce low amounts of ϵ chains (0.03 ± 0.02), which virtually disappear from 8 weeks onward. Synthesis of β -globin is 0.05 ± 0.02 at 7 weeks, and increases up to ~10% at 10 weeks.

To analyse the differential globin synthesis in primitive megaloblastic and definitive macrocytic cells, $>99\%$ pure megaloblasts were obtained from yolk sac at 5 weeks (Fig. 1a), as well as from heart and/or cord blood at 6–7 weeks (Fig. 3). At 5 weeks the megaloblasts synthesize essentially ζ - and ϵ -globin (Fig. 2b), and at 6–7 weeks a large amount of α chains and a small aliquot of γ -globin are also produced (~0.75 and 0.25, respectively) (for representative results see Fig. 3). Thus, primitive erythroblasts undergo a partial embryonic $\rightarrow$ fetal globin switch, the $\zeta \rightarrow \alpha$ transition apparently progressing more rapidly than the $\epsilon \rightarrow \gamma$ one. We observed no β -globin synthesis in primitive erythroblasts.

Definitive macrocytic erythroblasts have been purified from 6–7-week-old liver cells using a 7-fraction Percoll density gradient¹⁸. In a representative experiment, 6-week-old erythroblasts from fractions 3 and 4 (containing 93% and 83% macrocytic cells, respectively) synthesized respectively 0.24 and 0.28 of $\epsilon/\epsilon + \gamma$ chain (Fig. 4). At 7 weeks ϵ -globin represented

Fig. 2 a, Pattern of globin chain synthesis in yolk sac (YS) and liver (L) erythroblasts 5–10 weeks after conception. Values at 5 weeks were always identical in yolk sac and liver from the same embryo. Cells ($0.5\text{--}3.0 \times 10^6$) were incubated for 2 h at 37°C in air with $100\ \mu\text{Ci}$ of ^3H -leucine ($>100\ \text{mCi ml}^{-1}$; Amersham) in $0.5\ \text{ml}$ of leucine-free human dialysed AB plasma²⁰. Cells were washed three times in phosphate-buffered saline, and lysed in $10\ \text{mM}$ Tris-HCl pH 7.4, $5\ \text{mM}$ MgCl_2 . Haemolysates were recovered after high-speed centrifugation, freeze-dried and resuspended in small volumes of electrophoresis or isoelectric focusing (IEF) loading buffer. Triton-urea electrophoresis (TUE, right-hand panel) and urea-NP40 IEF (left-hand panel) were carried out on polyacrylamide gels as described elsewhere^{16,17}. $0.5\text{--}1.0 \times 10^5$ c.p.m. in $5\text{--}10\text{-}\mu\text{l}$ volumes were loaded on each slot. **b**, Time course of the $\zeta \rightarrow \alpha$ (left) and $\epsilon \rightarrow \gamma$ (right) globin switch in the overall pool of liver erythroblasts at 5–10 weeks. Relative globin synthesis ratios, as determined from densitometric tracing of IEF or TUE fluorographs, are indicated (mean $\pm$ s.e.m.).

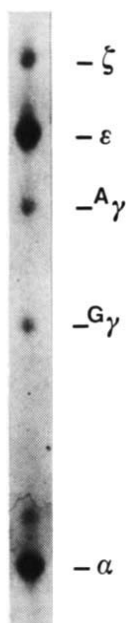
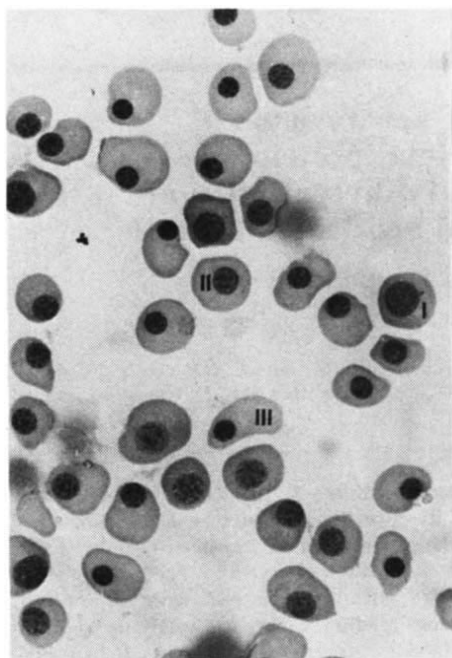
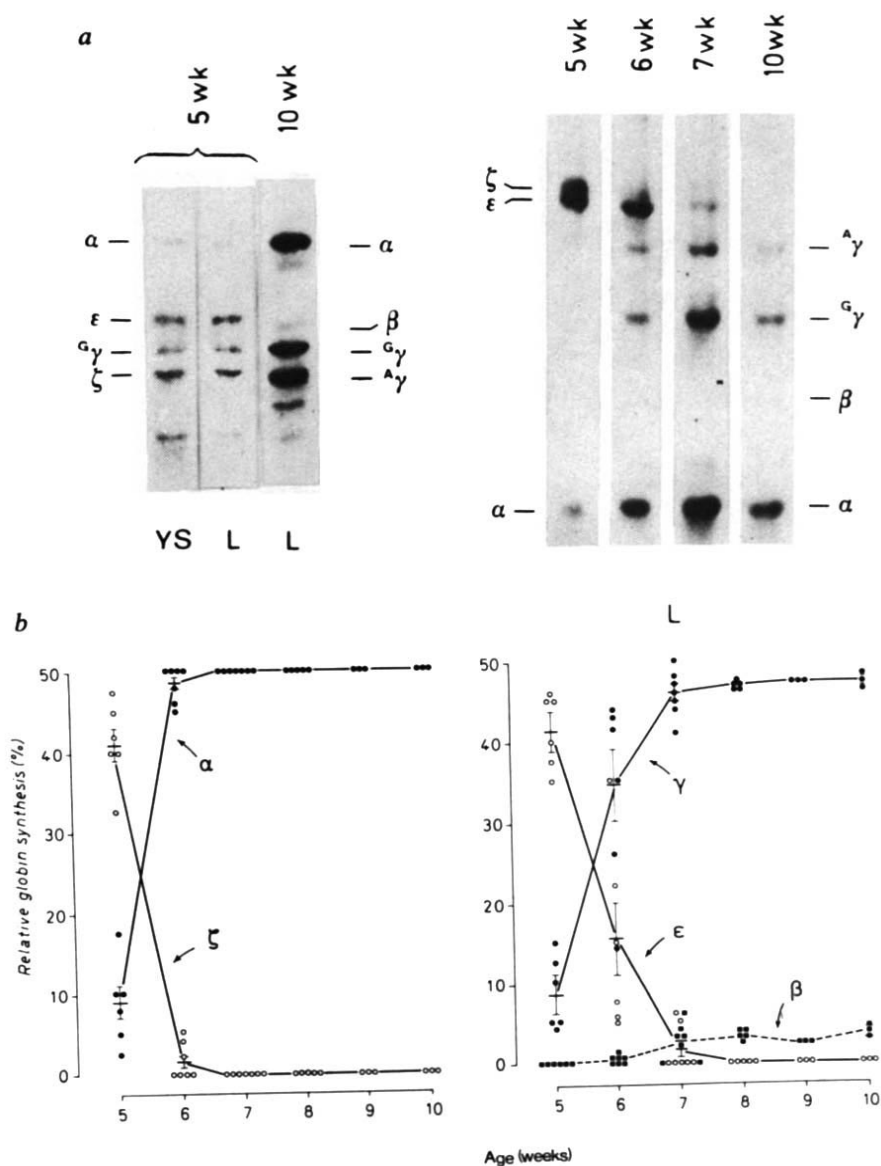


Fig. 3 Primitive lineage erythroblasts from peripheral blood at 6 weeks (left) and their corresponding pattern of globin synthesis (right: improved separation of ζ - and ϵ -globin was achieved by running the TUE gel for 30 h instead of 16 h, as in other experiments presented here). Contaminating definitive erythroblasts represented $<1\%$ of the total. Differential count: type I, 57%; type II, 39%; type III, 4% (see Fig. 1 legend).

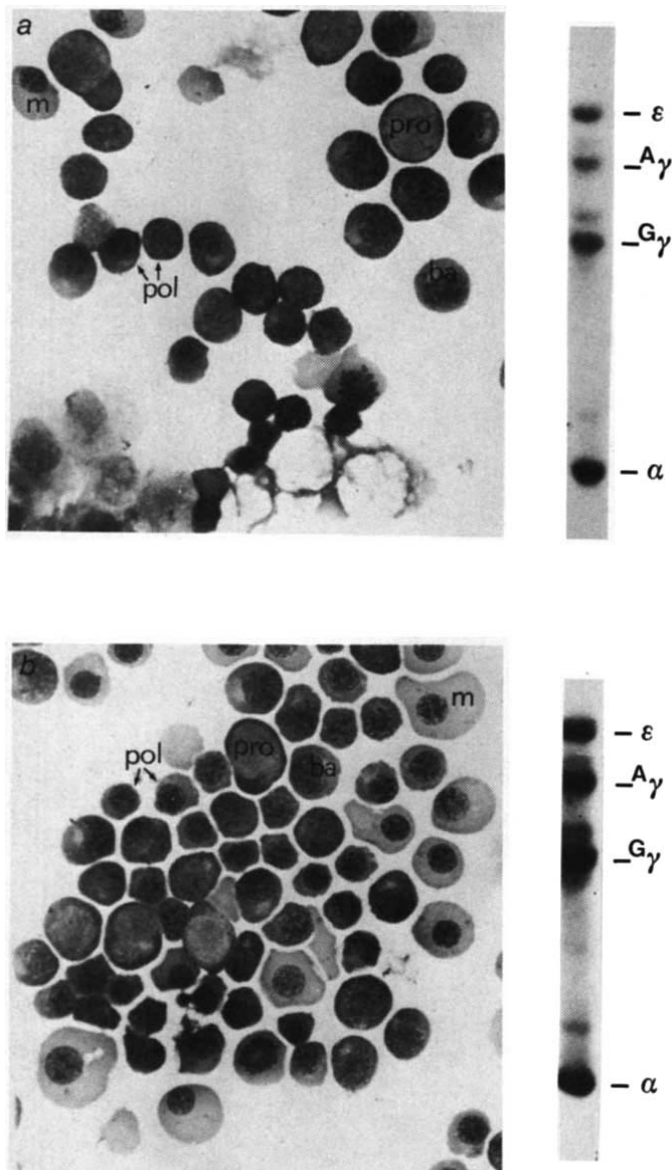


Fig. 4 Definitive lineage erythroblasts purified by Percoll density gradient centrifugation of a 6-week-old liver cell suspension (*a* and *b*, fractions 3 and 4, respectively); their corresponding patterns of globin synthesis are shown on the right. Differential count of fraction 3 (density = 1.066): proerythroblasts (pro), 13%; basophilic erythroblasts (ba), 52%; polychromatophilic erythroblasts (pol), 28%; primitive megaloblasts (m), 7%. Differential count of fraction 4 ($d = 1.078$): pro, 3%; ba, 41%; pol, 39%; m, 17%.

<0.08 in both fractions (not shown). In overexposed fluorographs of fractions 3 and 4, the β -chain was clearly present even at 6 weeks, whereas ζ -globin synthesis was always undetectable. Therefore, the ζ -globin gene is apparently switched off in definitive erythroblasts. Conversely, the ϵ -globin gene is still partially active at 6 weeks, but is gradually suppressed in the 7–8-week period.

In the primitive lineage of yolk sac origin, embryonic ζ - and ϵ -globin genes are thus fully active at 5 weeks, but become progressively suppressed at 6–7 weeks. Fetal α and γ genes, conceivably inactive before 5 weeks, are conversely activated in a balanced fashion. Activation of the α gene, however, occurs more rapidly than that of γ genes. The β gene is always silent. In definitive erythroblasts, the ϵ gene is still partially active at 6 weeks, but totally suppressed at 7–8 weeks; in parallel, the γ genes become fully activated, while gradual derepression of the β gene is initiated. The ζ gene is always fully suppressed, and the α gene totally activated. The 'lineage exclusion' of ζ - and

β -gene expression in macrocytic and megaloblastic cells respectively, indicates that α - and β -like gene clusters are regulated via at least partially different mechanisms.

These data obviously pertain to the cellular origin of erythropoietic lineages in early embryogenesis. We suggest that the embryonic $\rightarrow$ fetal haemoglobin switching occurs as a gradual continuum, and may be subdivided into two partially overlapping phases (the initial megaloblastic and the final macrocytic one). This phenomenon lends itself to a monoclonal model, whereby a single stem-cell pool undergoes in parallel a switch of the programme for both erythroid differentiation (megaloblasts $\rightarrow$ macrocytes) and haemoglobin synthesis ($\zeta \rightarrow \alpha$, $\epsilon \rightarrow \gamma$). This pool would first give rise to the primitive, limited 'burst' of yolk sac erythropoiesis, and then migrate to the liver, where it would give rise to the definitive erythroblastic lineage. Indeed, migration of stem cells from liver to bone marrow underlies the establishment of definitive adult erythropoiesis, which similarly implies a switch of both differentiation pattern (that is, macrocytes $\rightarrow$ normocytes) and haemoglobin synthesis ($\gamma \rightarrow \beta$)^{3,4,11}.

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Appearance of new variants of membrane skeletal protein 4.1 during terminal differentiation of avian erythroid and lenticular cells

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The erythrocyte plasma membrane is lined with a network of extrinsic proteins, mainly spectrin and actin, which constitute a reticulum tethered to the intrinsic anion transport protein of the lipid bilayer through a linker protein, ankyrin^{1–3}. Protein 4.1 forms a stable ternary complex with spectrin and actin^{4–7}, thereby strengthening the reticulum and anchoring it directly to the lipid bilayer⁸ or to another intrinsic protein, glycophorin⁹. It has been

found recently that spectrin¹⁰⁻¹⁴, ankyrin^{15,16} and protein 4.1 (refs 17-20) are not erythrocyte-specific; this has elucidated further the mechanisms of plasma membrane assembly and modelling during the differentiation of diverse tissues. We have shown previously¹⁸ that protein 4.1 in chickens is most abundant in erythrocytes and lens cells, but is scarce or absent from other spectrin-rich cell types. In addition, it exists as a family of related polypeptides showing differential expression in these two tissues, suggesting variant-specific functions. Here we show that the pattern of protein 4.1 variants changes during the terminal differentiation of erythroid and lenticular cells, with novel variants appearing in post-mitotic cells. The accumulation of these variants may lead to the final stabilization of the plasma membrane skeletons of these cells.

Protein 4.1 in mature chicken erythrocytes has six polypeptide variants with relative molecular masses (M_r) ~87,000 (87K) 100K, 115K, 150K, 160K and 175K (ref. 18), the 115K and 100K variants being predominant. A distinct set of variants, however, has been found in early embryonic erythroid cells. Circulating cells from embryos incubated at 38°C for up to 5 days are primarily primitive series erythroblasts^{21,22}. Immunoblot analysis using an antiserum specific for protein 4.1 (ref. 18) indicates that the major variant of protein 4.1 in these cells is 87K (Fig. 1, lane 1) with a novel variant of 77K, a 150K variant and a cluster around 100K. As the embryos develop further, variants characteristic of mature erythrocytes appear and begin to accumulate (Fig. 1, lanes 2-5).

Interpretation of these data in terms of erythroid differentiation is complicated by the appearance of the definitive series erythroid cells in the circulation at about 5 days of incubation. In contrast to primitive series cells, which disappear by about 16 days of incubation, definitive series cells represent the sustained lineage persisting throughout the life of the organism. There is, therefore, a mixed population of erythroid cells in the circulation between 5 and 16 days of incubation, with the number of definitive cells exceeding that of the primitive cells after about 9 days²¹.

As cells of these two lineages differ in size, however, they can be separated by velocity sedimentation²³ and analysed independently of one another. In the 7-day embryos, the 100 and 115K variants characteristic of mature erythrocytes begin to accumulate in primitive cells (Fig. 2, lanes 5-7), but are nearly absent from the definitive cells (Fig. 2, lanes 3, 4). The 7-day definitive cell pattern is thus similar to that of pre-5-day primitive cells. Similar analysis of 8-day cells reveals that the definitive cells have now begun to express the protein 4.1 variants characteristic of mature erythrocytes (data not shown). Thus, the ultimate change in the pattern of protein 4.1 variants is similar for both primitive and definitive series cells, although not coordinated temporally.

The appearance of the mature ('late') erythrocyte forms of protein 4.1 correlates well with the cessation of cell division. Primitive series cells, which develop as a cohort in the embryonic circulation, stop dividing at 5-6 days of incubation, whereas definitive series cells stop 2-3 days later (thereafter, only post-mitotic definitive cells are released into the circulation from sequestered sites of erythropoiesis)^{21,22}. This maturational difference between the two series suggests that the change in protein 4.1 variant expression is not triggered by organismal development, but is a function of the differentiation of the cell lines themselves.

To examine the kinetics of the protein 4.1 variant switch, circulating cells from a single 7-day embryo were analysed by immunoblotting and immunoprecipitation of protein 4.1 from ³⁵S-methionine-labelled cells. Immunoblotting detects total accumulated cellular protein, whereas immunoprecipitation reveals the polypeptides being synthesized at that instant. The two patterns of protein 4.1 variants thus obtained are similar, suggesting that the switch in the synthesis of the protein 4.1 variants is gradual (Fig. 3, lanes 1-4). Asynchrony in the cell population, however, prevents an accurate assessment of the kinetics of the switch in single cells; for example, the rate of shut-down of the synthesis of the 77K variant in individual,

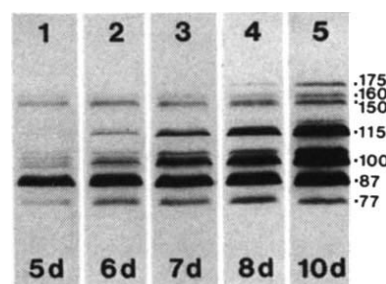


Fig. 1 Lanes 1-5, immunoblot analysis of protein 4.1 in circulating erythroid cells from individual chicken eggs at 5, 6, 7, 8 and 10 days of incubation. The embryos were at the following developmental stages as defined by Hamburger and Hamilton³³: 25, 28, 28, 32 and 35.5, respectively. Cells were lysed hypotonically in cold 5 mM MgCl₂, 1 mM EGTA, 4.4 mM Tris (pH 8.0), boiled in SDS sample buffer and sonicated to reduce viscosity¹⁸. Constituent polypeptides were resolved on a polyacrylamide slab gel and electrophoretically transferred to a nitrocellulose filter for subsequent analysis with anti-protein 4.1 antiserum and radioiodinated protein A¹⁸. The numbers on the right designate M_r values of the immunoreactive protein 4.1 variants.

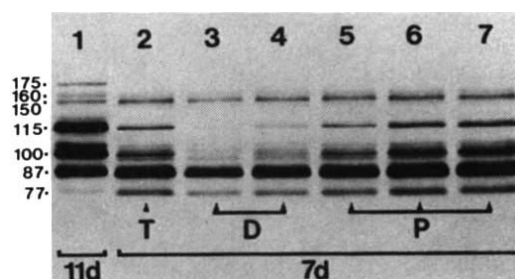


Fig. 2 Immunoblot analysis of protein 4.1 in erythroid cells from an 11-day (stage 36) embryo (lane 1) and from a pool of 7-day (stages 29 ± 1) embryos (lanes 2-7). The 7-day cells were size-fractionated by centrifugal elutriation; lanes 3-7 represent cells of increasing size. The 7-day cells were suspended in cold Hank's buffered saline solution containing 5 mM Na₂S₂O₈, 0.25% bovine serum albumin (BSA), no Mg²⁺ and no Ca²⁺, then loaded with a flow rate of 16 ml min⁻¹ into a Beckman JE-6B elutriator rotor spinning at 2,860 r.p.m.; fractions were eluted sequentially with flow rates of 20, 30, 36, 46 and 56 ml min⁻¹, respectively (lanes 3-7). These fractions contained either definitive (D) or primitive (P) series cells, as determined by their distinctive haemoglobin types (assessed by non-denaturing, pH 8.7 polyacrylamide gel electrophoresis^{21,23}; data not shown) and compared with the total (T) cell pool by immunoblotting with anti-protein 4.1. All cell samples were hypotonically lysed (as in Fig. 1, but with 10 mM 2-mercaptoethanol included in the buffer) and sedimented; supernatants were saved for haemoglobin analysis and cytoskeletal proteins in the pellet were solubilized with the same buffer containing 150 mM NaCl, 0.5-1.0% Empigen BB³⁴ at 0°C, cleared by centrifugation and boiled in SDS sample buffer. These immunoblots are indistinguishable from those of parallel samples of cells boiled directly in SDS sample buffer after hypotonic lysis. Lanes 2-7 each represent ~3 × 10⁶ cells. Similar results were obtained using unit-gravity sedimentation of cells through a BSA gradient²³.

post-mitotic cells may be much more rapid than in the population as a whole. Together with previous observations of the low level of synthesis of the 77K variant in circulating erythroid cells from 11-day embryos¹⁸ and virtual absence of this variant from erythrocytes in adult chickens¹⁸, the 77K variant seems to be either turned over and degraded in post-mitotic cells or diluted out by increased synthesis of the other 4.1 variants. In contrast, the 87K variant is synthesized and preserved as a major component of the membrane skeleton at all stages of differentiation.

We sought to determine whether a similar switching phenomenon could be observed and studied in an established

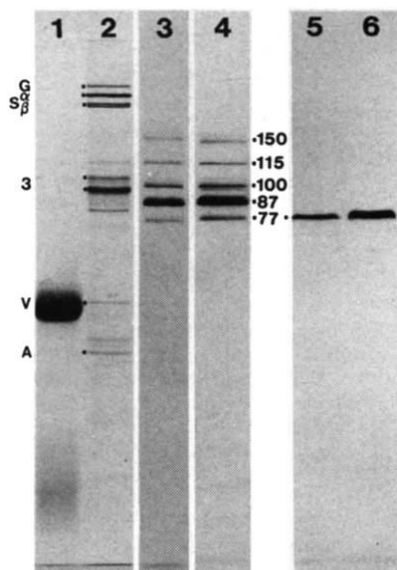


Fig. 3 Lanes 1–4, immunoprecipitation and immunoblot analysis of protein 4.1 from the circulating erythroid cells of a single 7-day (stage 30) chicken embryo. One aliquot of cells was labelled with ^{35}S -methionine and immunoprecipitated with anti-protein 4.1 essentially as described previously¹⁸, except that protein A-Sepharose (Pharmacia) was used as the immunoabsorbent. Coomassie blue staining of the immunoprecipitate (lane 1) reveals a trace of the 87K variant. Lane 2, mature chicken erythrocyte membrane proteins³⁵ for comparison. [G, globin (ankyrin¹⁶); S, α - and β -spectrin; 3, band 3, the anion transport polypeptides; V, vimentin (mobility similar to immunoglobulin G heavy chain in lane 1); A, actin]. Lane 3, fluorogram of lane 1, showing labelled variants of protein 4.1; lane 4, protein 4.1 immunoblot of another aliquot of the above cell sample, processed as described for Fig. 1. Lanes 5 and 6, protein 4.1 immunoblot of MEL cell cytoskeleton ($\sim 4 \times 10^6$ cells in each lane) before (lane 5) and 96 h after (lane 6) differentiation induced by adding 1.8% DMSO to the culture medium³⁶.

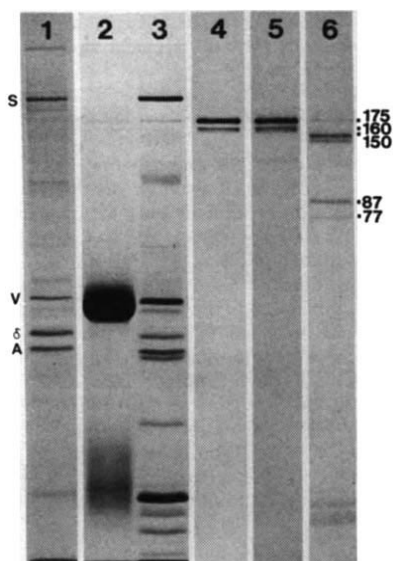


Fig. 4 Lane 1, fluorogram of hypotonically lysed and homogenized lenses from 12-day chicken embryos after labelling in organ culture with 200 μCi ^{35}S -methionine per ml of methionine-free RPMI 1640 for 4 h (S, spectrin; V, vimentin; δ , δ -crystallin; A, actin). Lane 2, Coomassie blue stained gel of protein 4.1 immunoprecipitate of this sample, showing the 175K and 160K variants; lane 3, adult chicken lens membranes²⁹ (Coomassie blue stained) for comparison; lane 4, fluorogram of lane 2; lanes 5 and 6, protein 4.1 immunoblots of chick lenses dissected into a cortical fibre fraction (lane 5) and epithelium fraction (lane 6); these samples were hypotonically lysed, homogenized and sedimented before solubilization for electrophoresis³⁰.

in vitro system. Murine erythroleukaemia (MEL) cells are transformed cells which can be induced to undergo limited erythroid differentiation with a variety of added agents, including dimethyl sulphoxide (DMSO)²⁴. In contrast to normal mouse erythrocytes, which possess two major variants to protein 4.1 (refs 18, 25), MEL cells have a single immunoreactive variant of 77K (Fig. 3, lane 5) of the same electrophoretic mobility as the smallest chicken variant, the same as the smallest ('4.1b') variant of normal mouse erythrocytes. Furthermore, this 77K MEL variant does not seem to change during the chemically-induced differentiation of these cells, although its quantity per cell increases significantly after 96 h in DMSO (Fig. 3, lane 6). Spectrin synthesis is also induced by this treatment²⁶.

Chicken lens fibres possess the same 4.1 variants as mature erythrocytes, but in vastly different proportions. The 175K and 160K variants predominate in lens fibres, whereas the smaller variants are virtually absent (ref. 18 and Fig. 4, lane 5). The thin layer of lens epithelial cells which proliferates and elongates to produce the lens fibres, however, shows a distinct pattern of 4.1 variants, with the 77K and 87K variants characteristic of proliferative erythroid cells, a doublet at 140–150K and a trace of the 175K variant (Fig. 4, lane 6).

Most of the epithelial cell mass of adult lenses is post-mitotic but actively elongating in an equatorial region (the annular pad). Differentiation of annular pad cells into lens fibres is the final elongation event responsible for continuous addition of new cells to the peripheral cortex of the lens throughout life^{27,28}; the immunoblots suggest that this is the stage at which the late (175K and 160K) variants of protein 4.1 begin to accumulate (Fig. 4, lanes 5, 6).

Immunoprecipitation of protein 4.1 from ^{35}S -methionine-labelled 12-day embryo lenses reveals that the 175K and 160K variants (characteristic of the fibre cells) are the predominant forms being synthesized (Fig. 4, lanes 1–4), correlating with immunofluorescence microscopy of frozen sections of these lenses (data not shown) and adult lenses¹⁸ where most of the protein 4.1 is in the fibres and very little in the epithelium. After about 4 days of embryogenesis, all of the fibre cells are post-mitotic but continue to elongate²⁷; thus, the late variants of protein 4.1 are synthesized apparently during this terminal elongation phase, perhaps stabilizing the membrane skeleton as it is being assembled.

Relative to the amount of spectrin present^{11,29,30}, the quantity of protein 4.1 in the lens epithelial cells is considerably less than in the cortical fibres; densitometry of immunoblots and Coomassie blue stained gels indicates that for a certain spectrin concentration there is approximately four times more protein 4.1 in the fibres than in the epithelial cells (data not shown), suggesting that the spectrin network in the epithelial cells is more malleable than in the fibres. This quantitative difference is superimposed on the contrast between the expressed 4.1 variants, which may also affect the properties of the membrane skeleton.

In conclusion, our data demonstrate that the expression of protein 4.1 changes during the post-proliferative, terminal differentiation stages of erythroid and lenticular cell development. Protein 4.1 shows a relative increase in concentration and an alteration in the pattern of synthesized variants, temporally correlated with a cessation of activities such as cytokinesis and endocytosis and with the establishment of a stable, permanent membrane skeleton in these cell types. Protein 4.1 may also function to prevent certain membrane proteins, such as glycoporphin, from being unnecessarily endocytosed during the final stages of differentiation³¹. In addition to the observed diversity of its variants, the presence of protein 4.1 in proliferative cells indicates that the different variants have different functions or binding capacities. We have seen no comparable changes in other cytoskeletal proteins during the differentiation of these cell types^{30,32}, suggesting that protein 4.1 represents a key regulatory element in the control of the cytoskeleton.

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Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells

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Human tumours often contain DNA sequences not found in normal tissues which are able to transform cultured NIH 3T3 cells. In some tumours the gene responsible for this transformation belongs to the cellular *ras* gene family^{1,2}. A specific type of mutation is responsible for converting the cellular proto-oncogene into a *ras* oncogene capable of inducing transformation³⁻⁵. In a study of the function of a cellular *ras* gene, its protein product (produced in a bacterial cell) was microinjected into NIH 3T3 cells; the recipient cells became morphologically transformed and were induced to initiate DNA synthesis in the absence of added serum⁶, but only when cellular *ras* protein was injected at much higher concentrations than required with protein of the transforming *ras* gene^{6,7}. To further analyse the function of the cellular *ras* gene, we have now injected monoclonal antibodies against *ras* proteins into NIH 3T3 cells. We report here that NIH 3T3 cells induced to divide by adding serum to the culture medium are unable to enter the S phase of the cell cycle after microinjection of anti-*ras* antibody, showing that the protein product of the *ras* proto-oncogene is required for initiation of the S-phase in NIH 3T3 cells.

In the present study we used monoclonal antibodies (238 and 259) against viral *ras* protein prepared by Furth *et al.*⁸ (obtained from D. R. Lowy). These antibodies bind both viral Harvey *ras* protein and the related cellular protein, but antibody 259 alone recognizes viral Kirsten *ras* protein⁸. In separate studies these antibodies were coinjected with purified *ras* protein into

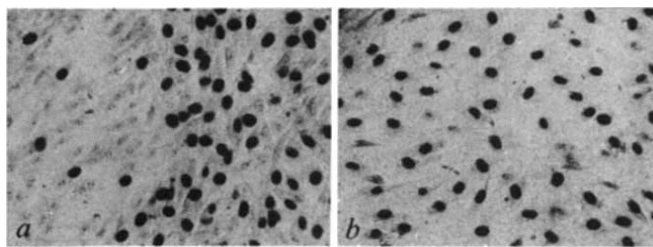


Fig. 1 Inhibition of DNA synthesis after microinjection of anti-*ras* antibody into NIH 3T3 cells. Antibody 259 (a) or 238 (b) was injected into NIH 3T3 cells located in the left half of each panel. Injected cells had been cultured in 0.5% FCS-containing medium for 24 h before injection; the cells were then transferred immediately to fresh medium containing 10% FCS. Tritiated thymidine ($5 \mu\text{Ci ml}^{-1}$, 41 Ci mmol^{-1} ; Amersham) was added to the culture between 12 and 24 h after antibody injection. The cells were fixed and autoradiographed as described previously⁶. Antibody 259 alone was able to block incorporation of label into the nuclei of injected cells. As a control, uninjected plates of cells were left in 0.5% FCS-containing medium but labelled with thymidine as above; <5% of these cells incorporated thymidine. Antibodies were purified from serum-free medium (Hana Media Inc., Berkeley, California) in which hybridoma cells had been grown. Immunoglobulin was purified by ammonium sulphate fractionation, followed by DEAE-Sephacel (Pharmacia) ion-exchange chromatography.

NIH 3T3 cells. Antibody 259 neutralized the coinjected *ras* protein, whereas similar amounts of antibody 238 did not reduce the ability of *ras* protein to transform the injected cell. Furthermore, the titre of purified antibody 259 against native *ras* protein was much greater than that observed with purified antibody 238 (H.-F. Kung, M.R.S., E. Bekesi, J. Manne and D.W.S., in preparation). Consequently, antibody 259 was used as a neutralizing antibody whereas antibody 238 or purified immunoglobulin against human interferon (given by H.-F. Kung) were used as negative controls.

NIH 3T3 cells were induced to enter the resting phase of the cell cycle (G_0) by incubation in medium containing 0.5% fetal calf serum (FCS) for 24 h, at which time no more than 5% of the cells were in S phase and synthesizing DNA. Purified immunoglobulin (10 mg ml^{-1}) was microinjected into the cytoplasm of as many cells as was technically possible (~ 100 cells) within a small circular area, and the culture treated with fresh 10% FCS-containing medium. Tritiated thymidine was added to the culture medium between 12 and 24 h after injection and autoradiography revealed that 70-98% of the cells in the culture had entered S phase. It was routinely observed, however, that only 5-15% of the cells in the area injected with antibody 259 had incorporated thymidine (Fig. 1a). When the efficiency of microinjection and serum starvation are taken into account, this result indicates that almost every cell injected during G_0 was blocked from entering S phase by the antibody. No inhibition of entry into S phase was observed for cells injected with either antibody 238 (Fig. 1b) or purified monoclonal antibody against human interferon (10 mg ml^{-1}). These results were duplicated with separate preparations of antibodies 259 and 238.

While immunoglobulin molecules are known to remain in an injected cell for several hours, microinjected proteins are removed eventually from the recipient cell⁹. Therefore, in the experiments described above, enough antibody had to be injected for there to be sufficient remaining after several hours to neutralize *ras* protein produced by the cell. To determine the amount of antibody required, several concentrations were tested. Efficient inhibition of thymidine incorporation (86%) was observed when antibody was injected at 7 mg ml^{-1} , whereas 2 mg ml^{-1} caused only partial inhibition (47%) and 0.75 mg ml^{-1} resulted in only 28% inhibition of thymidine incorporation in resting cells treated with 10% FCS-containing medium (data not shown). At 7 mg ml^{-1} , $\sim 3 \times 10^6$ antibody molecules would

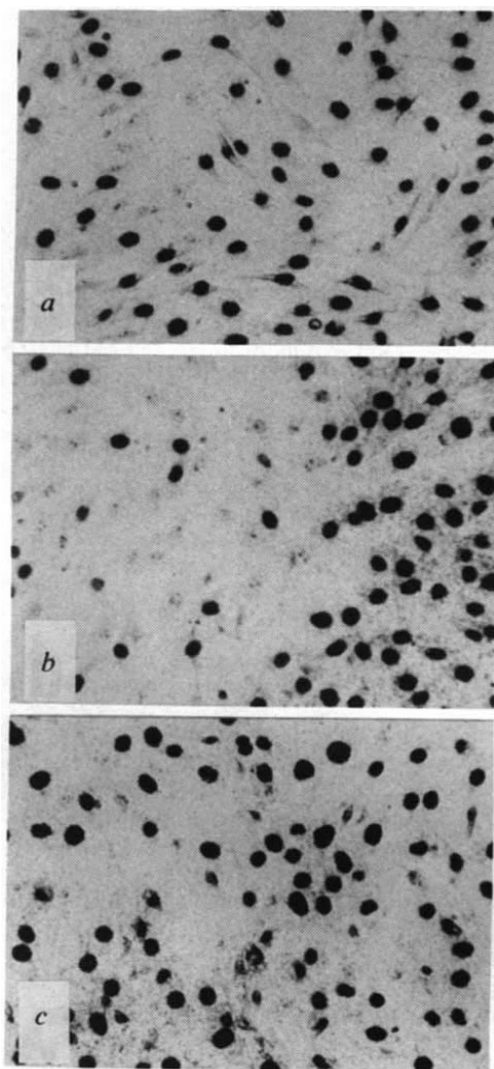


Fig. 2 Inhibition of new S-phase initiation in proliferating NIH 3T3 cells. The cells on the left half of each panel were injected with antibody; the other cells remained uninjected. The injected cells labelled during the first period (0–11 h, *a*) incorporated thymidine as efficiently as their uninjected neighbours. The injected cells labelled between 11 and 22 h after injection (*b*) showed inhibition of thymidine incorporation. Cells labelled at later times (22–33 h, *c*) exhibited uninhibited thymidine incorporation.

Methods. NIH 3T3 cells (1.0×10^5) were plated in a 35-mm culture dish 24 h before injection of antibody 259. Three separate cultures were injected and treated individually with labelled thymidine at 11-h intervals, after which they were fixed and autoradiography was performed.

be injected per cell; this would represent ~0.2% of the cell protein.

The requirements for induction of S phase may differ for resting cells (in G_0 phase) compared with actively growing cells (in G_1 phase); therefore antibody 259 was injected into cells of an actively-growing NIH 3T3 culture which would contain cells in all stages of the cell cycle. Injected cultures were pulse-labelled with thymidine at 11-hr intervals. (Judging from the 21-h doubling time of these cells and the number (~40%) of cells in S phase in a short thymidine pulse, we calculate that S phase lasts for 10 h, the same as for almost any cultured cell.) Little or no reduction in the number of cells synthesizing DNA was observed in the culture labelled for the first 11 h (Fig. 2*a*), but a dramatic reduction was observed between 11 and 22 h; thereafter, no effect of injected antibody was observed (Fig. 2*c*). As there was no apparent effect on thymidine incorporation immediately after injection, cells which had entered S phase

Table 1 Inhibition of thymidine incorporation by antibody injection at various times after serum stimulation of quiescent NIH 3T3 cells

Time of injection (h)	% Cells in S phase	No. of injected cells	% Inhibition*
0	3	40	86
4	2	53	53
6	1	101	62
8	14	119	35
10	49	150	24
13	69	168	15
15	75	169	18

NIH 3T3 cells were placed in medium containing 0.5% FCS for 24 h; at various times thereafter, fresh medium containing 10% FCS was added to duplicate plates. Antibody 259 was injected at the times indicated following serum addition. Labelled thymidine was added to the cultures immediately after injection until ~24 h following the initial addition of 10% FCS-containing medium. At the time of injection, labelled thymidine was added to the uninjected companion culture (which had been treated with 10% FCS at the same time as the injected culture) for 2 h to determine the percentage of cells in S phase. Fixation and autoradiography are described in Fig. 1 legend.

* The percentage of injected cells which failed to incorporate labelled thymidine. An average of 95% of the uninjected cells in these cultures was found to be labelled with thymidine.

before the injection presumably did not require *ras* activity to continue DNA synthesis. Most cells which had been in S phase at the time of injection would have completed DNA synthesis by 11 h after injection of antibody. Thymidine incorporation at this time would involve primarily cells which had entered S phase within 11 h post-injection. Significantly, only a few of the injected cells incorporated thymidine in this time period, indicating that the injected anti-*ras* antibody had blocked initiation of a new round of DNA synthesis. The injected cells appeared otherwise normal and healthy in this and in other experiments. At later times (that is, after 22 h), injected antibody apparently had been removed from the cells and normal DNA synthesis was observed among injected cells.

The data presented above indicate that serum-induction of cell division requires *ras* protein. To estimate the time at which *ras* protein acts, a time course analysis was conducted. NIH 3T3 cells were induced to enter resting phase by serum deprivation. At ~2-h intervals thereafter, fresh medium containing 10% FCS was added to separate plates of cells. At 15 h after the initial addition of serum, anti-*ras* antibody 259 was injected into cells of each culture and labelled thymidine added. In this way antibody was introduced into cells at various stages of the cell cycle, beginning at G_0 and extending into S phase. At the time of injection, duplicate plates of treated cells were pulsed with labelled thymidine for 1–2 h to determine the percentage of cells which had begun DNA synthesis in each case. The results (Table 1) indicate that *ras* protein is required just before the beginning of S phase, which was initiated ~8 h after the addition of serum to the cultures. Cells injected over 8 h after serum addition had begun DNA synthesis and were observed to incorporate label into their nuclei almost as well as neighbouring uninjected cells. At 8 h after addition of serum, 14% of the cells had already entered S phase and there was a 52% average reduction (three separate analyses; standard deviation 15%) in the number of labelled cells in the injected area of the culture compared with uninjected areas. At 6 h after serum addition, few cells were in S phase and the injected antibody effectively blocked subsequent entry into S phase. As before, these data indicate that anti-*ras* antibody cannot halt a cycle of cellular DNA synthesis that is in progress but it efficiently blocks entry into a new cycle of DNA synthesis.

Serum would be expected to contain a variety of growth factors. Peptide growth factors apparently either prime a cell for entry into S phase (a competence factor) or stimulate a competent cell to enter S phase (a progression factor; refs 10, 11). A purified competence factor (platelet-derived growth fac-

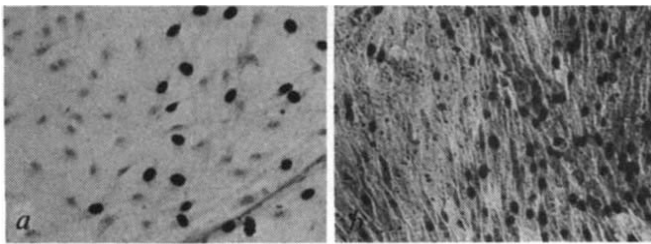


Fig. 3 Inhibition of DNA synthesis after microinjection of anti-*ras* antibody into NIH 3T3 (a) or MRC-5 (b) cells. a, NIH 3T3 cells cultured in 0.5% FCS-containing medium for 24 h and injected (in the left half of the panel) with antibody 259 were returned to their original medium following injection of antibody, and 25 ng ml⁻¹ PDGF (BRL) and 25 ng ml⁻¹ EGF (Sigma) were added to the culture. Cells were labelled and autoradiography performed as described in Fig. 1 legend. b, MRC-5 cells (American Type Culture Collection, passage 21–23) were plated at 3.0×10^5 cells per 35-mm tissue culture dish and injected 24 h later with antibody 259. The cells shown had been labelled between 15 and 18 h after injection.

tor, PDGF) and a progression factor (epithelial growth factor, EGF) were used instead of serum to stimulate quiescent 3T3 cells as above. Cells were induced to enter a resting phase by culture in 0.5% FCS-containing medium, then injected with antibody 259 and treated with 25 ng ml⁻¹ PDGF and 25 ng ml⁻¹ EGF; 54% of the treated cells were induced to initiate DNA synthesis by the combined action of the growth factors, but less than 10% of the cells receiving antibody incorporated thymidine (Fig. 3a).

NIH 3T3 cells are routinely transformed by a single oncogene^{12–14} and can be transformed by injecting *ras* protein^{6,7}, whereas MRC-5 cells (diploid, fibroblast-like human cells) behave like primary culture cells and are not affected by injection of *ras* protein. To determine the effect of treatment with anti-*ras* antibody on MRC-5 cells, several actively growing cultures were injected with antibody 259 and pulse-labelled with thymidine at various times thereafter. A culture pulse-labelled between 15 and 18 h post-injection showed a reduced level of thymidine incorporation (58% of the uninjected cells but only 13% of antibody-injected cells incorporated thymidine; see Fig. 3b), while cultures pulse-labelled at earlier times did not. In addition, antibody 238 had no effect on MRC-5 cells (data not shown). Thus, the action of *ras* apparently is not unique to one cell type.

Amino acid sequence data reveal the relationship of two oncogenes in one case to a peptide growth factor (PDGF)^{15,16} and in the other case to a growth factor receptor (EGF)¹⁷. In addition, the levels of transcription of several other proto-oncogenes are stimulated soon after serum addition^{18–22}. We have microinjected neutralizing antibody to the cellular *ras* protein to study its involvement in cell division. Microinjected immunoglobulin molecules remain within the cytoplasm of injected cells for several hours unless they encounter an antigen, at which time both may be cleared rapidly from the cell⁹. In this way the activity of a cellular protein may be transiently neutralized, providing a means of identifying its function within the intact cell (see ref. 23).

Here we have shown that the protein product of the *ras* proto-oncogene is required for NIH 3T3 cells to initiate the S phase of the cell cycle in response to stimulation by serum. Previous work demonstrated that the cellular *ras* protein could promote the initiation of S phase in the absence of added growth factors if injected in sufficient concentration⁶. As *ras* appears to be both sufficient and necessary for NIH 3T3 cell division, it must have an integral role in the control of cell division. Multiple cellular *ras* genes have been identified^{24,25}; in view of their critical role in cell division it is interesting that in many human tumours one of the cellular *ras* genes is mutated to a transforming oncogene.

The exact role of cellular *ras* protein in cell division is unknown; it seems to be a common requirement for the action of a variety of growth factors as no growth factor present in serum could promote efficiently cell division in NIH 3T3 or MRC-5 cells containing anti-*ras* antibody. Thus, the *ras* protein can be distinguished from the cellular protein analogous to middle-T antigen of simian virus 40, as the antibody to the latter only blocks partially cell division²³. The *ras* protein may, therefore, represent a common element in the molecular sequence initiated by numerous growth factors. Alternatively, cell division may involve a complex interplay of multiple intracellular signals, only one of which involves cellular *ras* protein. In either case, it is significant that *ras* protein apparently has a critical role shortly before the initiation of DNA synthesis.

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A retrovirus-like strategy for expression of a fusion protein encoded by yeast transposon Ty1

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Eukaryotic transposons such as the Ty element of yeast¹ or the *copia*-like sequences of *Drosophila*² show structural and functional similarities to both prokaryotic transposons^{3,4} and retroviral proviruses^{5–8}, but the prokaryotic transposons and retroviral proviruses use markedly different expression strategies which yield products having entirely different functions^{3–5}. To determine the phylogenetic relationship between eukaryotic transposons, prokaryotic transposons and retroviruses, we have sought to identify and characterize the proteins encoded by the yeast Ty element and to describe the strategies used to express these proteins. We show here that the yeast transposon produces a fusion protein by a specific frameshifting event that fuses two out-of-phase open reading frames (ORFs). The process is remarkably similar to that used by retroviruses such as Rous sarcoma virus (RSV) to produce Pr180^{gag-pol} (ref 5, 9).

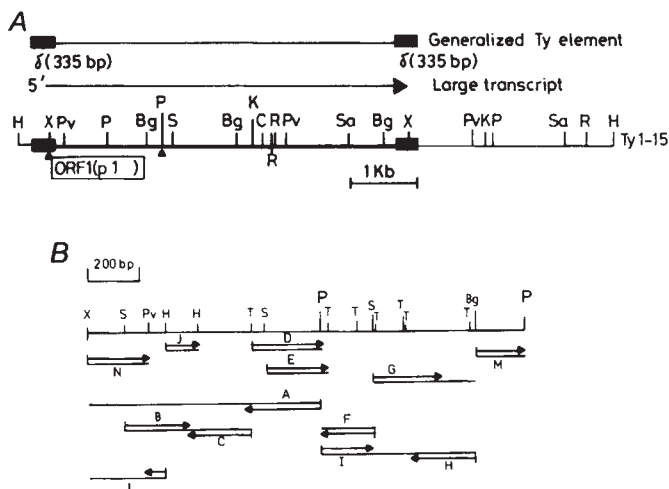


Fig. 1 **A**, Map of the 9.6-kb *Hind*III fragment containing Ty1-15. Solid boxes represent the delta sequences. The major transcript is indicated and is used as the standard for orientation of the element. The position of ORF1 encoding p1(Ty1-15) is marked. p1(Ty1-15) was previously designated p52(Ty1-15) (ref. 14 and J.M. *et al.*, in preparation). The fragment shown in **B** is marked by arrowheads. H, *Hind*III; X, *Xho*I; P, *Pvu*II; Bg, *Bgl*II; S, *Sac*I; K, *Kpn*I; C, *Cl*aI; R, *Eco*RI; Sa, *Sac*I. **B**, Sequencing strategy for the region around ORF1. Bars mark fragments that were subcloned into M13mp18 and M13mp19 (ref. 26) and the arrows mark the extent of the sequence read from those fragments. The dideoxynucleotide chain termination method was used²⁷. All reagents were from BRL and were used according to the manufacturer's instructions. X, *Xho*I; S, *Sau*3A; P, *Pvu*II; H, *Hae*III; T, *Taq*I; P, *Pst*I; Bg, *Bgl*II.

Ty elements are about 5.9 kilobases (kb) long with long terminal direct repeats (LTRs), delta sequences, of ~335 base pairs (bp)^{1,10,11}. The structure of a 9.6-kb *Hind*III fragment containing the element used in this study, Ty1-15, is shown in Fig. 1A. The major transcription product of Ty elements is a 5.7-kb transcript that extends from close to the *Xho*I site in the 5' or left delta sequence to 50 nucleotides beyond the *Xho*I site in the 3' or right delta sequence (Fig. 1A)^{12,13}. Recently, we have shown that Ty elements encode several protein species¹⁴. Figure 1A shows the location of the coding region of one of these, a basic protein of relative molecular mass (M_r) 52,000 (52K), designated p1(Ty1-15)¹⁴.

The genetic organization of the region encoding p1(Ty1-15) was analysed by determining the nucleotide sequence of Ty1-15 from the *Xho*I site in the 5' delta region to the second *Pst*I site (marked with arrowheads in Fig. 1A) (see Fig. 1B). The sequence from the initiation codon of the ORF running out of the 5' delta sequence¹⁵ to 30 nucleotides beyond the *Bgl*II site (Fig. 1B) is shown in Fig. 2. In reading frame 1 there is a long open reading frame, designated ORF1, running from nucleotides 1 to 1,320 (Fig. 2) that encodes a protein of 439 amino acids with a M_r of 50,426 and a calculated pI of 7.8; this confirms our previous observation that this region encodes a basic protein, p1(Ty1-15), of estimated M_r 52K¹⁴. Reading frames 2 and 3 have no significant open reading frames up to nucleotide 1,282 where an open reading frame, designated ORF2, starts in reading frame 2 with an ACA threonine codon. Therefore, ORF1 and ORF2 overlap by 38 nucleotides. Reading frames 1 and 3 lack significant open reading frames beyond nucleotide 1,282 to the second *Pst*I site shown in Fig. 1B, whereas ORF2 extends beyond this *Pst*I site (data not shown).

To determine whether ORF2 is expressed in Ty elements, an α_2 -interferon cDNA coding sequence was inserted into the *Bgl*II site at nucleotide 1,410, so that the interferon coding sequence was in-frame with ORF2 (Fig. 3). Expression of interferon was then used as a marker for ORF2 expression. As control construc-

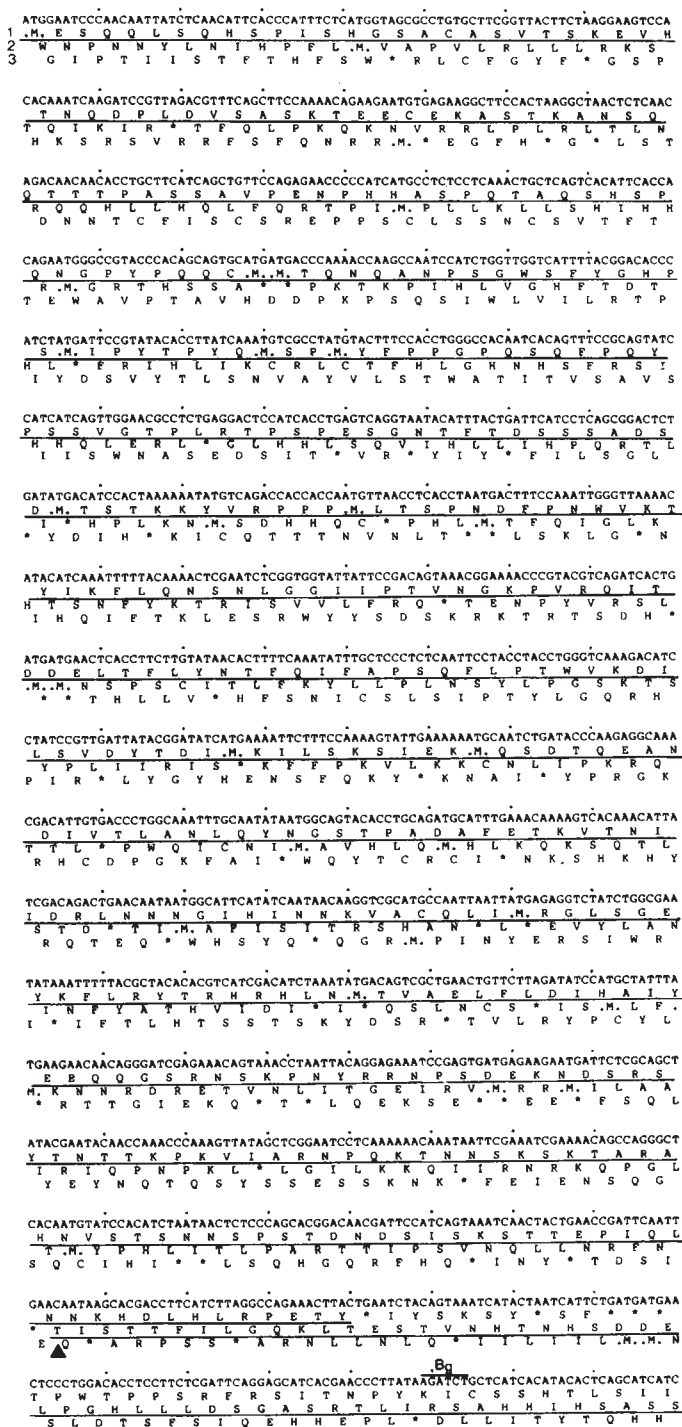


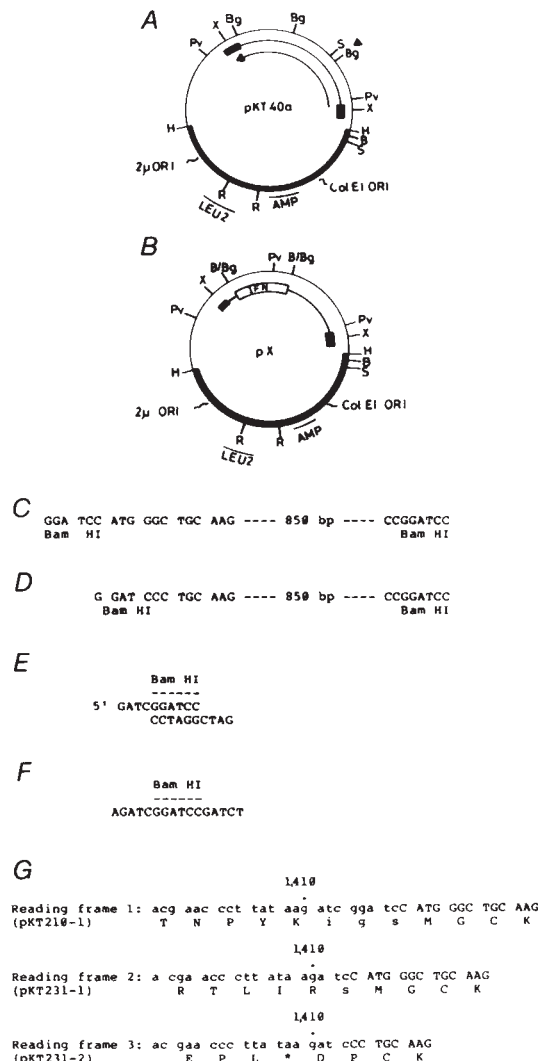
Fig. 2 Nucleotide sequence of the ORF1 region of Ty1-15. The sequence starts at the ATG at the beginning of ORF1¹⁵ and ends 157 nucleotides into ORF2. The reading frames are marked on the first line. ORF1 in reading frame 1 and ORF2 in reading frame 2 are underlined. The threonine residue at the beginning of ORF2 is marked with an arrowhead. * Termination codon; Bg, *Bgl*II.

tions, the sequence coding for interferon was also inserted in-frame with reading frames 1 and 3 (Fig. 3). Interferon was chosen because there are sensitive bioassays available and α_2 -interferon is a relatively small protein (20.8K) so that if it is produced as a fusion protein the size of the non-interferon component of the fusion can be determined relatively accurately. The starting molecule for these constructions was plasmid pKT40a (Fig. 3A) which is a yeast/*Escherichia coli* shuttle vector carrying the 9.6-kb *Hind*III fragment containing Ty1-15 (Fig.

Fig. 3 A, Map of plasmid pKT40a. Thick line, *E. coli*/yeast shuttle vector, pMA40 (ref. 15), sequences. pMA40 is a pAT153 (ref. 28) derivative containing the yeast *LEU2* gene and the 2 μ plasmid origin of replication, ORI²⁹. Thin line, 9.6-kb *Hind*III fragment containing Ty1-15 (Fig. 1A). The position of the Ty element and its direction of transcription are marked by a generalized Ty structure and an arrow. The *Bgl*II site at position 1,410 in Fig. 2 is marked by an arrowhead. pKT40a and its derivatives transform yeast at a high frequency ($10^5 \mu\text{g}^{-1}$) and they are maintained at a copy number of ~ 100 per cell¹⁷. H, *Hind*III; Pv, *Pvu*II; X, *Xho*I; Bg, *Bgl*II; S, *Sal*I; B, *Bam*HI; R, *Eco*RI; B/Bg, *Bam*HI/*Bgl*II junction. B, Map of the general structure, designated pX, of pKT231-1, pKT231-2 and pKT210-1. The α_2 -interferon coding sequence is shown replacing the two small *Bgl*II fragments in pKT40a. Two interferon fragments were used. Fragment 1 is a 0.85-kb *Bam*HI fragment originally described by Tuite *et al.*³⁰. Fragment 2 differs from fragment 1 only in the first 12 nucleotides which alter the relationship of the 5' *Bam*HI site to the interferon reading frame (see C and D). C, Structure of the ends of the *Bam*HI fragment 1 encoding α_2 -interferon. D, Structure of the ends of the *Bam*HI fragment 2 encoding α_2 -interferon. E, The structure of the *Bam*HI linker inserted at the *Bgl*II site at position 1,410 (Fig. 2) in pKT231. F, The resulting structure of the modified *Bgl*II site in pKT210. G, Ty/interferon junctions in plasmids pKT210-1, pKT231-1 and pKT231-2. Lower-case nucleotides are derived from Ty and linkers; upper-case nucleotides are derived from interferon *Bam*HI fragments. Upper-case amino acids are authentic Ty or interferon amino acids; lower-case amino acids are the result of linker insertions. * Stop codon. The nucleotide at position 1,410 (Fig. 2) is marked.

Methods. Initially two derivatives of pKT40a were constructed. Plasmid pKT40a was cut with *Bgl*II and religated to produce a derivative, pKT231, that lacks the two *Bgl*II fragments within Ty1-15. Plasmid pKT231 was then cut at the now unique *Bgl*II site and a *Bam*HI linker of the structure shown in E was inserted to create a modified, defective *Bgl*II site containing a *Bam*HI site (F); this derivative was designated pKT210. Interferon fragment 1 was then inserted into the *Bgl*II site of pKT231 and the *Bam*HI site of pKT210 to produce plasmids pKT231-1 and pKT210-1, respectively, and interferon fragment 2 was inserted into the *Bgl*II site of pKT231 to produce pKT231-2. DNA manipulations and plasmid DNA preparations were as described previously^{10,14,15,30}. All junctions were confirmed by nucleotide sequencing. These plasmids were used to transform¹⁶ yeast strain MD40-4C (*ura2*, *trp1*, *leu2-3*, *leu2-112*, *his3-11*, *his3-15*) to leucine independence.

1A). The general structure of the plasmids pKT231-1, pKT231-2 and pKT210-1 containing the interferon coding sequence is shown in Fig. 3B and the relationship of the interferon coding sequence to ORF1 and ORF2 is illustrated diagrammatically in Fig. 4A. Plasmid pKT231-1 contains interferon in-frame with ORF2 in reading frame 2 (Figs. 2, 3G). Plasmids pKT231-2 and pKT210-1 are control constructions with interferon in-frame with reading frames 3 and 1 (Figs. 2, 3G), respectively. The three plasmids pKT231-1, pKT231-2, pKT210-1 were used to transform¹⁶ yeast strain MD40-4C to leucine independence and the resulting transformants were designated T231-1, T231-2 and T210-1, respectively. Total RNA was isolated from these transformants and probed via a Northern blot with interferon fragment 1. Transcription should be promoted by the Ty signals in the 5' delta sequence, proceed through the Ty sequences up to the *Bgl*II site, through the interferon coding sequence, then terminate in the fortuitous yeast transcription terminator present at the 3' end of the interferon fragments¹⁷; this should result in Ty/interferon fusion transcripts of ~ 2.3 kb for all three constructions (Fig. 4A). The data in Fig. 4B show that this was indeed the case. All three constructions should direct the expression of pl(Ty1-15)¹⁴ but interferon expression should be dependent on the nature of ORF2 expression. Protein extracts of the parent strain MD40-4C and of T231-1, T231-2 and T210-1 were analysed by SDS-polyacrylamide gel electrophoresis and by a viral RNA reduction assay for interferon activity¹⁸. As predicted, similar amounts of pl(Ty1-15) were present in extracts of T231-1, T231-2 and T210-1¹⁴ (data not shown) but interferon activity (10^4 U per mg protein) was detected only in extracts of T231-1. This result shows that interferon is expressed only when it is



inserted in-phase with ORF2 in reading frame 2, suggesting that interferon is being expressed in T231-1 as an ORF2/interferon fusion protein. If this is the case, the provision of an initiation codon for the ORF2/interferon protein presents a problem as ORF2 begins with ACA(Thr) and there are no in-frame initiation codons between this codon and the *Bgl*II site at position 1,410 (Fig. 2). To gain some insight into ORF2/interferon expression, the size of the fusion protein was measured in Western blots provided with ¹²⁵I-iodinated antibody to α_2 -interferon (Fig. 4C). In agreement with the bioassay data, immunoreactive material was seen in the T231-1 track but not in the T231-2 or T210-1 tracks. One major and two minor bands corresponding to Ty/interferon fusion proteins (75K, 50K and 40K, respectively) were seen in the T231-1 track (Fig. 4C). Therefore, ~ 55 K, 30K and 20K of Ty-encoded protein was fused to interferon to produce these bands; this additional protein must be derived from ORF1 as there is no significant open reading frame having this coding capacity in reading frame 2 or 3 (Fig. 2). To produce the 75K fusion protein, virtually all of the coding capacity of ORF1 would be required, while ~ 900 and 600 nucleotides would be needed to produce the 50K and 40K proteins, respectively. In all cases this would require a frameshift event before the ORF1 termination codon to bring ORF1 and ORF2 in phase. An obvious mechanism by which these processes may occur is an RNA splicing event in the vicinity of the region in which ORF1 and ORF2 overlap. However, sequence searches have not revealed any yeast consensus RNA splicing signals^{19,20} and S₁ nuclease protection analyses have failed to detect any spliced RNA species (data not shown). The relationship of the three proteins in the Western blot is unclear but the 50K and 40K

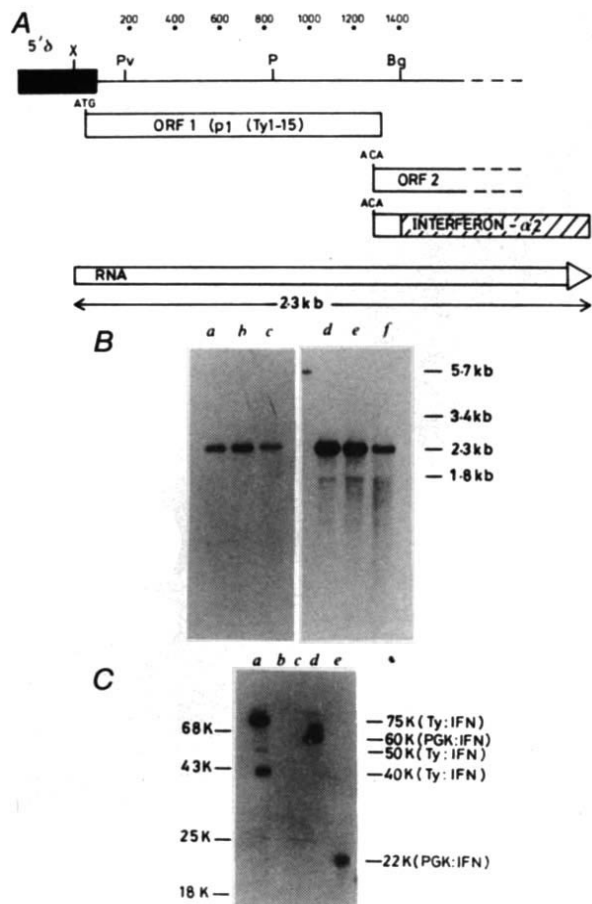


Fig. 4 A, The relationship of ORF1, ORF2 and ORF2/interferon fusion. The size and position of the predicted fusion transcript are shown. Numbers at the top represent the scale in nucleotides. B, Northern blot analysis of transcripts from pKT231-1, pKT231-2 and pKT210-1. Lanes a and d, pKT231-1; b and e, pKT231-2; c and f, pKT210-1. Lanes a-c were probed with the *Xho*I-*Bgl*II fragment containing ORF1 (Fig. 1) and lanes d-f were probed with the *Bam*HI interferon fragment 1. C, Western blot analysis of interferon fusion proteins in extracts of yeast transformants T231-1 (lane a), T231-2 (b), T210-1 (c), T361-1 (d) and T230-1 (e). All sizes are in kilodaltons. T361-1 and T230-1 are MD40-4C transformants containing plasmids pMA361-1 and pMA230-1 (ref. 30) which encode phosphoglycerate kinase/interferon fusion proteins of relative molecular mass 60K and 22K, respectively (J.M., unpublished data). Extracts of these transformants were included as positive controls for the Western blot.

Methods. A, B, Probe fragments were labelled to 2×10^8 c.p.m. μg^{-1} by nick-translation³¹. Total RNA was isolated as described previously¹⁴, transferred to nitrocellulose filters and hybridized³². C, Protein extracts of whole yeast cells were prepared as described previously³³. SDS-12.5% polyacrylamide gels (30:0.8 acrylamide/bisacrylamide) were run according to Laemmli³⁴. After electrophoresis, the gel was rocked gently in blotting buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% (v/v) methanol) for 30 min. The blot was set up as follows: a sandwich composed of buffer-saturated 'Scotch-Brite' pad, 3MM paper, polyacrylamide gel, 0.45 μm nitrocellulose (BA85; Schleicher & Schuell), 3MM paper and Scotch-Brite pad was inserted into a BioRad Trans-Blot apparatus and run for 3 h at 70 V with the nitrocellulose facing the anode. After electroblotting, the filter was placed in two changes of low-salt blocking buffer [20 mM Tris pH 7.5, 0.9% (w/v) NaCl, 0.05% v/v Triton X-100, 0.5% casein (Hammersten, BDH)] for one period of 30 min. The filter was then placed in a sealable plastic bag and incubated overnight at room temperature (with the protein-bearing side of the filter towards the bench) with 1 ml of buffer (40 mM Tris pH 7.5, 1.8% (w/v) NaCl, 0.1% Triton X-100 and 1.0% radioimmunoassay grade bovine serum albumin) and 1 ml of monoclonal antibody (mouse NK2 anti-interferon antibody iodinated with ^{125}I , 12.6–19.5 μCi μg^{-1} , 7×10^5 d.p.m. ml^{-1}). The filter was then washed in four changes of low-salt blocking buffer (except that the casein was omitted) for 1 h with gentle shaking, dried and exposed to X-ray film at -70°C for 2 days.

species may be cleavage products of the 75K protein (J.M. *et al.*, in preparation).

The similarity of vertebrate retroviruses and eukaryotic transposons such as Ty1 has been noted (for example, see refs 5 and 21) and in *Drosophila* this structural analogy has been taken further by the discovery of copia RNA in virus-like particles²². In this study we extend these comparisons to the expression strategies of these elements. ORF1 and ORF2, in Ty1-15, are in the same relative positions as the *gag* and *pol* coding sequences in retroviruses^{5,9,23} (Figs 1, 4A). Retroviral *pol* regions are expressed as large precursor proteins by fusion of the *gag* and *pol* sequences. In RSV the *pol* open reading frame overlaps *gag* by 43 nucleotides and is in a different reading frame⁹, therefore either an RNA splicing event would be required to alter the reading frame to bring *gag* and *pol* in phase, or a frameshift suppression-like mechanism might be involved. There exists no direct evidence for any form of splicing event in or around the *gag-pol* fusion region of any retrovirus, but in Moloney murine leukaemia virus (Mo-MuLV), in which *gag* and *pol* are separated only by the amber termination codon of *gag*²⁴, direct evidence for a nonsense suppression-like mechanism has been obtained (Y. Yoshinaka, I. Katoh, T. D. Copeland and S. Oroszlan, personal communication). The organization of ORF1 and ORF2 in Ty1-15, and their expression as a fusion protein via a frameshift event, is remarkably similar to *gag-pol* organization and expression in RSV-type retroviruses (Figs 2, 4). We suggest, therefore, that the frameshift event described here is common to Ty and retroviruses such as RSV. In the absence of any evidence for RNA splicing and given direct evidence for a suppression event in Mo-MuLV, it is possible either that the mechanism is a function of the primary or secondary structure of the RNA interfering with ribosomal recognition processes or that the elements have evolved to take advantage of low-level endogenous frameshift suppressors²⁵. Whatever the mechanism, the tractability of yeast as a molecular genetic system will allow a detailed analysis of the problem.

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Contaminated komatiites

HUPPERT *et al.*¹ calculate that Ni ores in ultrabasic lavas could be formed by assimilation of sulphidic bedrocks and Nisbet² comments that models based on komatiite chemistry may be spurious artefacts of contamination. Here, we draw attention to field and geochemical aspects of komatiites strongly suggesting that melting of their bedrocks did not occur.

Ni-ores are rare in komatiite lavas and, if the ground-melting model were correct, areas where none occur should correlate with low abundances of sulphidic sediments. This is not so: in Western Australia, for example, such sediments are ubiquitous and have frustrated countless geophysically-targeted searches in barren komatiite piles. Thus, assimilation of surface sediments cannot be a major mechanism of Ni-sulphide genesis.

Support for thermal erosion theories comes from Kambalda, where a sediment marking the komatiite/basalt contact is absent in ore-bearing troughs³. However, 'hanging-wall' ores above the troughs form consistently-aligned vertical stacks of deposits³ and the repetitive occurrence of ore-bearing flows, over the same area of sea floor, cannot be caused by assimilation, as the initial flow would deplete this area in sediments. The systematic pattern is more consistent with lavas flowing into pre-existing channels, as delineated elsewhere by sediments underlying komatiites³⁻⁶. Furthermore, there is little evidence for incipient melting or a thermal aureole in such sediments and there is a conspicuous absence of veining of bedrocks and of xenoliths in komatiite flows.

Surface-melting is distinct from other models for magma contamination in its selectivity; trace elements in each flow should reflect variable amounts of contamination from a wide range of possible substrates. However, it is difficult to explain observed systematic trends⁷⁻¹⁰ in such terms and, in particular, the anomalous Sm-Nd isotopic data for Kambalda¹¹ are unlikely to reflect selective contamination because the tight grouping of both komatiites and basalts on the same isochron is too coherent. Sulphur isotopes in Kambalda ores cannot distinguish between surface and other crustal sulphur¹² but chalcophile-element depletions show that all komatiites were saturated with sulphur before eruption¹³, which argues convincingly against selective assimilation of sulphur by ore-bearing flows.

That komatiites flow turbulently and so transfer heat efficiently seems inescapable. However, the chilled crust at the base of komatiite lavas^{14,15} is free of cumulate olivine and so must have formed during the turbulent phase when olivines were in suspension and when melting of bedrock would otherwise be most efficient. We sug-

gest that the basal crust develops in a manner similar to that at the flowtop (equation 7 in ref. 1) and that it forms an effective insulating layer between the ground and overlying hot, turbulent lava.

With no komatiites being erupted for us to observe today, theoretical treatments of what could occur are of great value. Nevertheless, it is apparent that the real lavas were more restrained and less voracious than their mathematical counterparts and this should impose a constraint on the physical properties to be modelled.

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HUPPERT *ET AL.* REPLY—Claoué-Long and Nesbitt present only some of the facts supporting thermal erosion and contamination in komatiites. Interpretations of these rocks are rarely straightforward, as their lavas are deformed and metamorphosed; primary volcanic features have only been well documented in a few places. However, we believe that Claoué-Long and Nesbitt may have failed to appreciate several aspects of our model¹, causing them to misinterpret some of the geological observations which they cite; we note specifically the following:

(1) Nickel ores are found commonly in komatiites²⁻³ and there is a general connection between komatiite-associated ore occurrences and sediments in the Norseman-Wiluna Belt of Western Australia⁴ and those in the Abitibi Belt, Ontario⁵. As in all ore deposits, a nickel-enriched mineralized locality must be complemented by much larger volumes of barren lava; only in this sense are the ores 'rare'.

(2) For Kambalda, there are several arguments favouring thermal erosion additional to those selected by Claoué-

Long and Nesbitt (see, for example, ref. 6). Contrary to the given impression, inter-flow sediments do occur in komatiites at the same stratigraphic levels as the hanging-wall ores but are absent from the ore zones. Furthermore, it is not unreasonable for successive lavas to follow the same path, the previous flow forming a depression by partial drain-out or sagging above its thickest part and subsequent komatiites accentuating the depression by thermal erosion.

(3) The thermal aureole at the base of a channel would only be a few cm thick. Given the sheared and regionally-metamorphosed condition of many contacts and the limited number of detailed studies, we are not surprised that evidence for an aureole or for xenoliths has not yet emerged and we emphasize that thin aureoles should be present whether or not the komatiite has eroded its floor rocks.

(4) Variations in incompatible element ratios in komatiites have been interpreted⁷⁻⁹ in terms of mantle heterogeneities but small amounts of contamination can produce similar effects. Thus we do not accept that contamination will necessarily produce non-systematic trends and emphasize the efficiency with which floor rocks would be homogenized during thermal erosion and assimilation.

(5) We are puzzled by Claoué-Long and Nesbitt's statement that the Nd isotopic data from Kambalda are anomalous. Claoué-Long *et al.*¹⁰ interpret these data as defining an old, but acceptable isochron; others¹¹ have suggested that the Sm-Nd array is a mixing line, perhaps resulting from contamination of komatiite with older granitic or sedimentary rocks, an interpretation consistent with thermal erosion.

(6) Chalcophile-element depletion provides evidence of sulphide fractionation but not as to where, when or how it took place.

(7) The reason why basal crust should form while the flow is moving is not clear to us. If basal chills prove to be a general feature of komatiites (and only two examples have been described) they will form at a late stage in the lava's history when turbulent motions have weakened. The boundary conditions at the base and top of a flow are fundamentally different as sea water can remove heat efficiently by convection whereas rock is a poor conductor. We estimate elsewhere¹² that any initial chill on the base will be dissolved away in a lava with prolonged throughput, which is precisely what is observed at Kambalda where massive sulphide overlies footwall basalt with no intervening komatiite chill, a puzzling feature until explained by thermal erosion.

Komatiites are highly susceptible to contamination effects. This may be inconvenient, but geologists may have to get used to the idea in the future.

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Cyclic GMP and neurone death

THE recent report by Weill and Greene¹, indicating that the daily treatment of chick embryos with dibutyryl cyclic GMP during the period of naturally occurring motoneurone death prevents much of this normal cell loss, is of considerable interest. For, if correct, this observation advances the analysis of neurone death by bringing us one step closer to understanding the cellular and molecular events involved. However, for reasons described below, I wish to provide a note of caution to those who, on the basis of this report, may intend to pursue this line of investigation.

In a review paper on neurone death published a few years ago, I suggested that cyclic GMP might be involved in the regulation of this developmental phenomenon². In fact, at that time a graduate student in my laboratory was investigating this problem³. Although his experiments were generally similar to those of Weill and Greene, he failed consistently to find any effect of cyclic nucleotides, including dibutyryl cyclic GMP, on neurone death. At about the same time, I was contacted by Dr Weill and asked to review a manuscript she was considering submitting for publication; this paper transpired to be virtually the same as that published later in *Nature*. On reading the paper, and through additional correspondence and conversations, I learned that there were a few procedural differences between our experiments and those of Weill and Greene: for example, although our experiments used several different doses of dibutyryl cyclic GMP, extending over a range of three log units, the highest dose was slightly lower than the single

dose used by Weill and Greene (that is, 0.91 mg). Consequently, since that time we have conducted four additional independent and virtually exact replicates of the Weill and Greene experiments, including the use of dibutyryl cyclic GMP from the same manufacturer's lot (Sigma, St Louis) and at the same dosage. In all four experiments the results were the same: dibutyryl cyclic GMP had no effect on motoneurone survival (day 10 controls = 12,192 ± 809, $n = 12$; day 10 experimentals = 12,356 ± 937, $n = 18$). We also failed to observe any differences in the number of nucleoli in the motoneurons of treated embryos, contrary to the report of Weill and Greene.

As the specific strain of chicken eggs used in the two laboratories differed, it is possible that genetic factors are at issue here. I believe, however, that this is highly unlikely: first, the four attempts by us to replicate the results of Weill and Greene fortuitously involved four different strains, including both 'egg' and 'broiler' types; second, we have never observed previously such all-or-none strain differences in a series of related pharmacological studies on neurone death⁴. Thus, in an extensive attempt to meticulously replicate the experiments of Weill and Greene, I have not been able to confirm their claim that dibutyryl cyclic GMP affects motoneurone survival.

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WEILL REPLIES—Our initial experiments in which we injected dibutyryl cyclic GMP into developing chick embryos were carried out using a crossed strain of chicken eggs (Harco × Partner). We found that a daily dose of 0.91 mg of dibutyryl cyclic GMP effected a 58% increase in the number of surviving lateral motor column motoneurons. Owing to the novelty of the observation, I contacted Dr Oppenheim and several other senior workers in the field and, at their collective suggestion, extended our original observations to include many more embryos. The results of this work were those published¹.

Subsequent attempts to repeat this experiment using White Leghorn embryos (Truslow Farms, Chestertown, Maryland) at the same dose level of dibutyryl cyclic GMP failed to increase the level of motoneurone survival. Further experimentation has revealed that daily injections of 0.864 mg of dibutyryl cyclic GMP.2H₂O effects the survival of 53.5%

of those cells that would have died by embryonic day 10 (day 10 controls = 14,390 ± 439, $n = 12$; day 10 dibutyryl cyclic GMP-treated = 17,950 ± 376, $n = 8$, mean ± s.e.m.). The dose-response curve is biphasic and the range of effective doses uncommonly narrow, as variations in dose of ±5% do not give a positive response. We observed no increase in the number of cells with two or more nucleoli in the White Leghorns, unlike the effect on the red strain. We have no explanation for the apparent difference in the response of the two strains. These experiments are nearing completion and a manuscript is in preparation.

Our interpretation of the results observed with the crossed red strain of embryos stemmed from our other published work², which demonstrated an increase in neuronal cyclic GMP levels over embryonic days 5–10 in the absence of a change in the cyclic GMP level of leg muscle over the same time period. An increase in neuronal cyclic GMP levels was also observed for spinal cord neurones co-cultured with muscle factors. We concluded that muscle factor(s) could cause an increase in neuronal cyclic GMP levels over the period of natural motoneurone cell death. We then wondered whether increased intraneuronal concentrations of cyclic GMP would enhance motoneurone survival by mimicking the presence of the appropriate muscle factors. Our interpretation of the results obtained is that dibutyryl cyclic GMP increases the neuronal levels of cyclic GMP and, via an as yet undetermined mechanism(s), effects an increase in motoneurone survival.

It was not our intention to slight the extensive contributions that Dr Oppenheim had made to this field by not including a discussion of our results in the context of his proposed model³, which involves intramuscular cyclic GMP levels. We had data demonstrating that levels of cyclic GMP in muscle did not change during natural motoneurone cell death, whereas intraneuronal cyclic GMP levels did increase during natural cell death². A discussion of the intramuscular cyclic GMP model did not seem germane to our conclusions. We are, in fact, indebted to Dr Oppenheim and his colleagues, for it is their work in part that has formed the basis for our own investigations.

Note added in proof: Meriney *et al.*⁴ have reported the prevention of ciliary ganglion cell death by cyclic GMP.

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Pilgrim's progress

Eric Ashby

Green Politics: The Global Promise. By Fritjof Capra and Charlene Spretnak.
Hutchinson/E.P. Dutton: 1984. Pp. 238. £10.95, \$11.95.

Seeing Green: The Politics of Ecology Explained. By Jonathon Porritt.
Basil Blackwell: 1984. Pp. 249. Hbk £15, \$24.95; pbk £3.95, \$6.95.

COME to think of it, it's not surprising that chlorophyll and bacteria are becoming symbols of a new faith. Industrial nations spend vast sums on ways to secure energy, a problem green plants had solved in the Palaeozoic. And these same nations are deeply concerned about the disposal of waste, a task bacteria can perform on all but a few man-made substances. For a long time biologists have been interested in photosynthesis and biodegradation; it is only recently, when energy sources seemed to be at risk and waste polluted the biosphere, that these processes became objects of veneration. Hence the Ecology Party in Britain and the Greens in West Germany.

Genuine ecologists (I used to be one of them) resented the hijacking of the name of their discipline by a bunch of what seemed to be "woolly-minded lentil stirrers" (the quotation is from Jonathon Porritt's book). What would come next? A Petrology Party? An Ethology Party? But the label sticks and the best thing to do is to find out what these born-again environmentalists are doing under cover of the word "ecology". These two books are as good an introduction as one could hope to get.

Many people — even those who voted for Mrs Thatcher or President Reagan — have apprehensions about the future of the life-style of capitalist systems. They have to live with disturbing paradoxes: food surplus in Europe, starvation in Africa; in Britain sewers collapsing, families desperate to get housing, inner cities in decay, and millions with no work to do; a money market which, if all debts were to be called in, would collapse overnight; rumours, never confirmed but never denied either, that permanent damage may be done to the air above us and the seas around us. Socialist systems offer no solution to these paradoxes: with differences in detail, they seem headed for a similar social insolvency. In Britain it is the credibility of a two-party system, adversarial, perpetually bickering, which is in question. People are coming to fear that neither side has a solution to the problem of how to manage a post-industrial society: is it not time to invent new political machinery to replace an obsolete nineteenth-century mode of government?

In Britain the response to this disquiet has been impressive: the creation of a new political party which, if we had proportional representation in the franchise,

would already have a significant representation in Parliament. In West Germany, too, there has been a response. In March 1983, supported by two million votes, 27 Greens took their place in the Bundestag of the Republic. They received 5.6 per cent of the total vote, with no support (indeed with active hostility) from the corporate interests of the nation, its industry and trade unions.

It is easy to say what the Greens (and their counterpart in Britain, the Ecology Party) are against. It is not so easy to say what they are for, at any rate not in terms that can be translated into practical politics. These two books try, and with some success, to deal with this common criticism; they should, if they do no more, silence the sneers that Greens are "messianic", "far-left", "volatile". Greens don't yet have the confidence to be messianic and most of them reject the far-left as vigorously as any Tory does. What they are certainly not, is volatile. They have come to stay, and we had better try to find out what they do stand for.

Capra and Spretnak are Americans who have already written about the life-style of their own country. For this book they went to Germany to find out all they could about the Greens. The result is a record of hundreds of interviews and research into documents and reports. They begin with a perceptive study of the way the Greens evolved from the confused protests of the 1970s. In those days the protests were inspired by Marxism. The extremists split away into careers of terrorism (the Baader-Meinhof group). The Marxists returned to the safe fold of Communism. Meanwhile, growing up around this core of discontent, there was in Germany a massive movement of people concerned with the environment, with nuclear disarmament, with women's rights, with a hunger for participation in

the decisions being made over their heads by their elected representatives, with non-violence. Out of this conglomerate of opinion the foundations of the Green Party were laid.

The authors make it clear that there was no easy consensus, apart from unanimity that many present values in industrial society are not to be tolerated. At their founding convention the delegates had to persuade the janitor to stop the clock so that the meeting could go on after the official closing time, in the hope that basic principles could be agreed upon. After bitter wrangling a form of words was accepted. There were to be "four pillars" of belief: ecology, social responsibility, grassroots democracy, non-violence. In the interpretation of these words there can be so many shades of green that to be a paid-up Green may mean that you are a vegetarian who abhors battery farming, or a CND activist, or an advocate of permissive abortion, or a campaigner against acid rain. Capra and Spretnak do not conceal the weakness of this broad tolerance; it is not very wise to go into battle against multinational corporations, entrenched governments, and greedy and affluent consumers, when each foot-soldier is out against his own personal enemy!

However, let the critics keep silent for a while: the Green Party is not yet five years old; its members are working desperately hard to strengthen the "four pillars" by giving meaning to them and by building upon them some superstructure of practical politics. The rest of Capra and Spretnak's book describes these efforts, and predicts the influence they may have on a Green Movement in the United States. How far have the German Greens got in creating a "philosophy" for Greens? This question is best answered by turning to Jonathon Porritt's book, for he has learnt much from the Greens in Germany.

Jonathon Porritt is a leading member of the Ecology Party in Britain and was recently appointed Director of Friends of the Earth. His book is an affirmation, written with clarity, modesty and sincerity. It is in two parts: a reasoned challenge to existing ideologies, and an attempt to replace them by ideologies acceptable to those who share his deeply held convictions; in brief, a diagnosis and a prescription.

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Jonathon Porritt (centre) and the Ecology Party's Council at the annual conference, 1983.

tion for treatment. It is a very personal book, so much so that even his critics (and there will be many of them) will read what he says with respect. My own snap-verdict is that the diagnosis is likely to prove correct but that at this stage of the disease the prescription will not work, for reasons I shall give in a few moments. First, the diagnosis.

On page 18 Porritt lists 14 things which it is shocking to find in a rational civilized society. They are all familiar (for example that the safeguard for keeping peace is to threaten to annihilate the planet) and old

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Talking to America: Petra Kelly, one of the founders of the Green Party, on the stump in Washington in 1983.

men like myself have become so familiar with them that the conscience becomes blunted to their enormity. What an outrage it must be for a young man like Porritt to find himself in a world where such things are condoned. Porritt's "dirty dozen", as he calls them, are to be found in scores of doomsday books, going back to the shoddy and sensational *Blueprint for Survival*, published 13 years ago. What is fresh and refreshing about Porritt's affirmation is his optimism and his David-and-Goliath determination to go on fighting to eliminate them, even though the odds are heavily against him in the short term. No sign of withdrawal into complacent communes or of distancing himself from the present political system. He is a pilgrim and he will continue his pilgrimage, whether you follow him or not, into the "enemy's" camp, even if that is Parliament.

So he sets out his objectives, which are the objectives of the Ecology Party. In doing so he puts strength into the "four pillars" described in the book by Capra and Spretnak.

Ecology: to have a holistic attitude to all the rest of Nature, animate and inanimate (he is critical of the assumption in *World Conservation Strategy*, that Man is both apart from Nature and part of Nature: for him, Man is part of Nature and no more).

Social responsibility: to get rid of the alienation that is a legacy of the Protestant work ethic; he works towards a society where what we would now call underemployment is called leisure, and where unemployment (denying the right of persons to work) has withered away; a great extension of self-help, too, with voluntary community service replacing much that is now done by the State. He

works, too, for inducements to bring people back, part time, on to the land (he is strongly in favour of people owning their own land) to grow some food for themselves.

Grassroots democracy: this, translated into politics, has plenty of precedent, from the New England Town Meeting to the assembly in an Ibo town in Nigeria. In the present political climate in Britain it is unnecessary to emphasize the rising resentment of people who are not consulted before decisions are made, and whose limited influence in local government is being ruthlessly eroded. He quotes what is, to me, the grimmest warning in the book: "... when a people begin to question the right of their leaders to govern, the leaders question the right of the people to question".

Non-violence: here nothing new, but no less important on that account. There is a mass-insanity about an arms race, which one doesn't have to be a Green to be ashamed about.

But what is the prescription for ridding the industrial nations of these evils? It is at this stage that the weasel-word "must" creeps in, on page after page. Applied to individuals, yes, it's valid. I "must" do what I believe to be right, whether I profit from it or not. But when "must" is applied to governments, multinational corporations, the totality of populations in a nation, the word is meaningless — unless "must" is to mean compulsion, arrest, labour camps, torture, the firing squad (and even with all these, there will always be men and women to defy "must"). It is a principle of the Greens in Germany that "all notions of dictating to the majority of people what is good for them are foreign to us". So Porritt, like the rest of us, is faced with the question: can you change human nature? He sweeps aside the cautious reply of those who have tried to do just that, and

he hopes (some hope!) that one day self-interest and the public interest will coincide. Well, one day they will, but not, I'm afraid, in the way Porritt hopes. It seems that human beings, although they can foresee environmental changes in a way other creatures cannot, nevertheless are not capable, as whole communities, to adapt their life-style in anticipation of the coming change. They can adapt only after the change has begun to operate and disaster stands at the door. Did we adapt to anticipate the industrial revolution? No. Are we showing any sign of adapting to the consequences of the present post-industrial revolution? None that I can discern.

There is one ground for optimism. It is drawn from the wisdom of R.H. Tawney, whom Porritt courageously quotes and who (I guess) is one of Porritt's heroes. First (on p. 124):

...since even quite common men have souls, no increase in material wealth will compensate them for arrangements which insult their self-respect and impair their freedom.

And (on p. 195):

...no change of system or machinery can avert those causes of social malaise which consist of egotism, greed, or quarrelsomeness of human nature. What it can do is to create an environment in which those are not the qualities which are encouraged.

On p. 216 Porritt sets out in two parallel columns the politics of industrialism and the politics of ecology. They cast a cold light on the task of changing human nature by peaceful persuasion. But — and this is why I was cheered by the book, although I disagree with many of its assertions — Porritt and others of his generation are not dismayed by the prospect. His motto could well be the words attributed to Columbus: "no land in sight, but sailed on". □

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What price nuclear power?

Michael Webb

Nuclear Power: Futures, Costs and Benefits.

By Nigel Evans and Chris Hope.
Cambridge University Press: 1984.
Pp. 171. £15, \$29.95.

ALL METHODS of generating electricity are associated with potentially harmful effects on human life and the environment. Yet, rightly or wrongly, many people see the possible hazards stemming from nuclear power stations as of a different order of magnitude to those connected with other forms of generation.

In recent years concern over nuclear power stations has been fuelled by various mishaps, especially the accident in 1979 at

Three Mile Island (TMI). The stated purpose of Evans and Hope's book is to consider how doubts about the costs and risks of nuclear power have grown and how they *should* (my italics) be taken into account when making decisions about future options for power generation. In their analysis, however, the authors explicitly ignore issues of proliferation, waste disposal, environmental effects (other than on human health) and the purely political aspects of nuclear power.

The book can conveniently be divided into three parts. The first (Chapter 2) reviews nuclear power programmes outside communist areas and estimates the total installed nuclear capacity up to the year 2000. It shows how such estimates have been continually revised downwards in recent years. The second part (Chapters 3–5) considers the possible frequency of core meltdown accidents in light water reactors (LWR) and the possible costs of such accidents. Finally, Chapters 6–10

deal with the analysis of risk and uncertainty in the appraisal of nuclear power projects.

Various methods can be used to estimate the risks to public safety stemming from accidents at nuclear power stations. One is the probabilistic risk assessment (PRA) method which was used by the US Nuclear Regulatory Commission in its report published in 1975 (variously known as the Rasmussen or WASH-1400 report). Instead of estimating frequencies of occurrences based on operating experience, PRA estimates frequencies based on judgements of what could go wrong, together with the consequences, and the likelihood of a particular accident. Using this method, WASH-1400 suggested that the 50% chance of a core meltdown was once every 20,000 reactor years. Evans and Hope estimate frequencies on the basis of aggregate operating experience to the end of 1982 and, interpreting the TMI accident as a meltdown, obtain a most likely value for frequency of core meltdown in LWRs as once every 858 reactor years. The authors correctly conclude that on their evidence "informed observers begin to feel uneasy" (p. 71). But their result is crucially dependent on the interpretation of TMI as a core meltdown, and that has not been clearly established.

The choice between alternative forms of power generation poses many problems, but one basic requirement is to ensure that all the relevant options have been considered. In Chapter 7 the authors examine the choice of the least-cost generation option to meet projected electricity demand in England and Wales, and show this to be a pressurized water reactor (PWR). Subsequently, however, they consider briefly possible trade in electricity between France and England, and show this to be a lower cost option than the PWR; the range of options covered in Chapter 7 is thus incomplete.

Conservationists will be interested in the authors' method of evaluating risks of nuclear power projects. The method uses weights based on value judgements to rank six options in terms of various risks, including risk of death, morbidity and of dread (such as dread at the thought of a nuclear accident). Their results show that, in terms of risk attributes considered and weights used, energy conservation is the preferred choice.

This is a thought-provoking book which should be of interest to both proponents and opponents of nuclear power. One of its main merits is that it does encourage the reader to think carefully about a number of issues. But it is regrettable that Evans and Hope were unable to apply their methods of analysis to nuclear waste management or to consider the risks associated with proliferation. □

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Alternative cultures

L.H. Jones

Handbook of Plant Cell Culture. Vol. 1 Techniques for Propagation and Breeding. Vol. 2 Crop Species.

Edited by D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada.

Macmillan, New York/Collier

Macmillan, West Drayton, Middlesex:

1984. Vol. 1 pp. 970. Vol. 2 pp. 644. \$53, £55 each volume.

Cell Culture and Somatic Cell Genetics of Plants. Vol. 1 Laboratory Procedures and Their Applications.

Edited by Indra K. Vasil.

Academic: 1984. Pp. 825. \$85, £59.50.

THE potential of tissue culture as a tool in plant breeding was made manifest in the late 1950s, with the first recovery of plants from culture. The power of rapid clonal multiplication of elite selections from the plant breeders' conventional crosses was soon recognized and resulted in the rapid development of clonal propagation methods, based mainly on micropropagation from excised buds or shoot tips. During the 1970s, the possibilities of using culture methods themselves as an extension of breeding techniques excited the popular press to exotic claims of the imminent appearance of super-crops derived from protoplast fusion and other cell manipulations. Despite the wild speculations, little of material value appeared in the seed catalogues as a result. But the underlying detail of techniques was being laboriously developed, and we thereby gained valuable experience in use of haploids, protoplasts and cell suspensions.

During and since that time a steady stream of papers appeared, describing new medium formulations and protocols for the propagation of an ever-widening range of species. Most tissue culture conferences became tedious recitations of recipes, frequently impossible to reproduce in other laboratories. Success or failure depends on so many subtle factors, that the whole process of plant tissue culture seems more a matter of empirical art than of science. So it was and so it remains. Now we have two multi-volume works that attempt to draw some sort of order from the chaos.

In the *Handbook of Plant Cell Culture*, to be published in five volumes of which two are currently available, D.A. Evans and his co-editors have attempted to bring together a really practical series of books, giving detailed references and full protocols for various culture techniques. The editors have drawn on distinguished authors from many different countries and have maintained a remarkably uniform style and format. Volume 1 concentrates on techniques for propagation and breeding, and gives a full literature review, species lists and most valuable, detailed protocols. These will be particularly useful

to the many workers who wish to use tissue culture as another tool in a complex programme rather than as an end in itself. Space was also found for two chapters on secondary metabolite production. This is perhaps the weakest part of the book; the subject matter is not in tune with the main theme and the coverage is not as comprehensive as that devoted to the plant breeding topics. There is for instance no mention of the important developments using immobilized cell systems. So much activity is taking place that this area, too, could stand a detailed treatment; perhaps the editors plan to do so in a later volume.

Although in the first volume many agriculturally important species are mentioned in passing, Vol. 2 deals specifically with individual crops, including many important species. More will be covered in Vol. 3. Each species is introduced with a short account of its history and economic importance, and a discussion of breeding problems, before we proceed to a survey of the tissue-culture literature and the detailed protocols.

Naturally, however, there are imperfections. The print and general layout are crowded and unattractive, and most chapters are so packed with detail as to make the books very definitely works for reference and not for general reading. Many of the reviews are unduly descriptive in nature and the reader might have been helped to sort out the most important papers by a few more selective value judgements in the text (this deficiency is remedied to some extent by a short list of key papers at the top of each bibliography). Although detailed protocols are given for most procedures, the contributors could also have given us more information on the vital stages of stock plant preparation and sampling which usually tip the balance between success and failure.

These, however, are carping comments on a remarkable compendium which can have missed little of the published literature. It is a generally successful attempt to produce a definitive statement of work on plant tissue culture techniques, and if the remaining volumes are of the same standard the series will be an invaluable reference work in tissue culture laboratories throughout the world. My copy of Vol. 1 is already well thumbed, and has developed a strong tendency to walk from the office desk to the laboratory bench down the corridor. There can be few higher recommendations for a book.

The first volume of the series from Academic Press (apparently planned as a "closed treatise") deals with laboratory procedures in cell culture and somatic cell genetics of plants, so can be viewed as being in competition with the Macmillan series. It

● Motoo Kimura's *The Neutral Theory of Molecular Evolution* has been issued in paperback by Cambridge University Press. Price is £12.50, \$19.95. For review see *Nature* 306, 713 (1983).

is, however, quite different in concept and presentation, although both series have many authors in common. The contributors do not attempt the comprehensive literature survey provided by their counterparts in Evans *et al.*, but present helpful "how-to-do-it" instructions with a good selection of important references. A wide range of practical methods is covered in 85 short, easily read chapters and little difficulty should be experienced in following most of the methods given, although many of them appear deceptively simple. The style of text and layout is more attractive than its rival, and while the two series have much subject matter in common there are many topics specific to each. Because it appears less confusing this book will appeal more to the newcomer to the field, but once started the reader may well wish to refer to the more detailed account of the literature given in the Macmillan series. If the budget will stretch, there is sufficient difference in subject matter, style and content to warrant purchase of both sets of volumes. □

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After Olive

Brian Shaffer

The Dictyostelids.

By Kenneth B. Raper.

Princeton University Press: 1984. 453 alkaline pages. \$97.50, £80.70.

ABOUT thirty years ago, the only slime moulds vaguely known to the average biologist were the myxomycetes. Since then the cellular slime moulds, which alternate between a unicellular and a multicellular life, have invaded even the elementary biology course as a paradigm of intercellular communication, adhesion and behaviour; and as waves of amoebae have swept in with what, not only in time-lapse photography but in real time, is such obliging rapidity, and then undergone binary differentiation in specific proportions to produce structures of specific shape, hundreds of biologists have been swept into studying them.

Aggregation in the larger species of dictyostelids is a relay system in which the central amoebae secrete an acrasin, which first attracts other cells and then induces them to secrete acrasin, so that they in turn attract and induce more peripheral cells. In some respects this is a model for much cultural transmission in human societies — the spread of technology, religions and isms, of FRS's and Nobel prizes. So it was perhaps surprising that it took quite a few years for my demonstration of this relay system to be generally accepted and for it then to induce further work on the relay

mechanism. Be that as it may, Professor Kenneth Raper was clearly one of the two or three founders of modern research into cellular slime moulds. One can easily trace the transmission of his influence to many laboratories, often by the students he attracted who in turn attracted others.

Since 1930 Raper has studied morphological development and taxonomy, a number of his techniques remaining unchanged. Whereas many workers restrict themselves to whatever standard medium is currently in use, Raper has always been concerned with the minute details of the slime moulds' natural environments and the effect of cultural conditions on development and hence on features used in classification. The allocation of space in this book reflects this emphasis. Nearly half is devoted to systematics, much of it dealing with each species' requirements; and the rest of the book, which gives a synoptic view of development, contains large sections on occurrence, isolation, ecology, cultivation and culture maintenance. This leads to some duplication of material but the fullness of treatment could convince an amateur naturalist that without high technology he could still usefully contribute to slime-mould studies.

While several recent books have dealt with different aspects of slime moulds, this is the first account since E. W. Olive's at the turn of the century to give a comprehensive description of all known dictyostelids. Raper characteristically maximizes his predecessor's contributions, probably previously entirely unknown to most present-day workers. Throughout, he refers generously to his colleagues, correcting their errors gently without polemic — as well as acknowledging his own. He avowedly gives minimal coverage to biochemistry and genetics.

The chapter on macrocysts may seem overburdened with somewhat confusing details from the original papers, but this is the long-missed sexual phase which should be of great importance for genetic and developmental analysis if details of culture receive sufficient attention. (All but the most interlinear reader may be baffled by the phrase "macrocysts neither observed nor reported" repeated in descriptions of several species.)

In this era of the bestseller, in which interest is concentrated on a tiny fraction of the available range of a particular subclass of object — whether the class be books, films, records or species — it is no surprise that almost all work on the cellular slime moulds has been confined to a single species, *Dictyostelium discoideum*, discovered by Raper himself. Here he is trying to redress the balance. One hopes that his hawking a much wider range of species than are usually on display will tempt other researchers into working on them. □

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Physical chemistry of proteins

Wayne L. Mattice

Proteins: Structures and Molecular Principles.

By Thomas E. Creighton.

W. H. Freeman: 1984. Pp.515. \$26, £28.95.

THOMAS Creighton aims this book at an audience with "a background in biology, with some knowledge of biochemistry, genetics, and cell biology". His readers are assumed to have no special expertise in the physical sciences.

Accordingly, six out of the ten chapters (about 75% of the book) discuss various aspects of the physical chemistry of proteins. These chapters — "Physical Forces that Determine the Properties of Proteins", "Conformational Properties of Polypeptide Chains", "The Folded Conformations of Globular Proteins", "Proteins in Solution", "Interactions with Other Molecules" and "Catalysis" — are clear and informative, and should prove interesting to the intended readership. Most of the remaining 25% is devoted to chapters entitled "Chemical Nature of Polypeptides", "Protein Biosynthesis" and "Evolutionary and Genetic Origins of Protein Sequences". As one expects of a book emanating from W. H. Freeman, the written word is supported by a great many illustrations of high quality.

Each chapter is copiously referenced, and the references are appropriate for the material presented. However, many of the papers concerned are at a level demanding a high degree of sophistication in physical chemistry, or a closely related field, and will be incomprehensible to a reader whose training is solely in biology.

The book contains occasional errors and contradictions. For example at one point it is asserted that dichloroacetic acid is a helix-supporting solvent. And in Chapter 5 it is stated that "Pro residues are incompatible with both the α -helix and β -sheet conformations", while the next chapter contains a table showing that Pro has a significant occurrence in both conformations. This contradiction could have been remedied by a short discussion of the role played by Pro at the amino terminus of helical segments and at the edges of sheets.

But the good points clearly outweigh the minor defects. For those with a biological background who would like to become familiar with the properties of proteins at the molecular level, Creighton's book will prove very helpful. □

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